STRUCTURE/FUNCTION ANALYSIS OF C/EBPα AND C/EBPβ

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ABSTRACT

CCAAT/enhancer binding proteins (C/EBPs) are modular transcription factors that play important roles in energy metabolism. For example, C/EBP transcription factors are important regulators of the expression of phosphoenolpyruvate carboxykinase (PEPCK), a key regulatory enzyme in gluconeogenesis in the liver. In support of this, C/EBP knockout analysis and antisense methodologies have uncovered a role for C/EBPα in mediating the cAMP responsiveness of the PEPCK gene promoter. The work in this thesis is dedicated to defining the regions within C/EBPα and C/EBPβ that mediate the constitutive and PKA-inducible activity towards the PEPCK promoter.

Examination of the regions of C/EBPα and C/EBPβ previously characterized to mediate a robust PKA-inducible response identified three conserved regions (CRs). Deletion analysis, in conjunction with GAL4 methodology and dominant negative transfection analysis, showed that CR2 (amino acids (aa) 61-70) was critical for mediating the PKA-inducible activity when C/EBPα occupied the distal sites on the PEPCK promoter.

However, CR3 (aa 96-106) and amino acids 6-50 of C/EBPα were found to be important for mediating the PKA-inducible response when it bound to the cAMP Response Element site (CRE). In contrast, the CRs within C/EBPβ did not appear to contribute to the PKA-inducible activity regardless of where it bound to the PEPCK promoter.

GST-pull down analysis indicated that aa 6-50 and CR3, but not CR2, were required for C/EBPα to physically interact with TATA-binding protein (TBP) and Transcription Factor II B (TFIIB). This protein-protein analysis, in conjunction with transient transfection analysis, suggested that the domains within C/EBPα that mediate the constitutive and PKA-inducible activities vary depending on which *cis*-element it occupies on the PEPCK promoter. For example, when C/EBPα binds to the CRE site, amino acids 6-50 and CR3 may mediate the constitutive and PKA-inducible activity through a mechanism that may involve physical interaction with TBP and TFIIB. However, when C/EBPα binds to the distal promoter region, CR2 may mediate the PKA-inducible response by interacting with different proteins, possibly a co-activator. The data suggest that the precise location of a transcription factor on a promoter may influence which domains within the protein are employed to mediate transactivation.

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H.L. Wilson, P.J. McFie, and W.J. Roesler, 2002. Identification of a Co-Regulator for C/EBPα. Am. Soc. for Biochemistry & Molecular Biology, Transcriptional Regulatory Mechanisms (Satellite meeting symposium/posters.) April 19-24, 2002, New Orleans, LA. (Abstract #4316)

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Deus diligat nos et conservet nos.

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LIST OF ABBREVIATIONS

1,4-Piperazine Diethane Sulfonic Acid, Sodium Salt	(PIPES)
2,5-Diphenyloxazole	(PPO)
3'-5'-Cyclic Adenosine Monophosphate	(cAMP)
Activator Protein-1	(AP-1)
Adenosine Monophosphate	(AMP)
Adenosine TriPhosphate	(ATP)
Amino Acid	(aa)
Base Pair	(bp)
Basic Region-Leucine Zipper	(bZIP)
Bovine Serum Albumin	(BSA)
cAMP Response Element	(CRE)
cAMP Response Element Binding Protein	(CREB)
cAMP Response Unit	(CRU)
Calcium Chloride	($CaCl_2$)
Carbon Dioxide	(CO_2)
CCAAT/Enhancer Binding Protein	(C/EBP)
Chloramphenicol Acetyltransferase	(CAT)
CREB Binding Protein	(CBP)
Conserved Region	(CR)
Curie	(Ci)
D-Binding Protein	(DBP)
Double Distilled	(dd)
Diethylpyrocarbonate	(DEPC)
Dimethylsulfoxide	(DMSO)
Dithiothreitol	(DTT)
Deoxyribonucleic Acid	(DNA)
DNA Binding Domain	(DBD)
Deoxynucleotide Triphosphates	(dNTPs)
Escherichia coli	(*E.coli*)
Ethylene-Diamine Tetraacetic Acid Disodium Salt	(EDTA)
Guanosine Diphosphate	(GDP)
Guanosine Triphosphate	(GTP)
General Transcription Factors	(GTFs)
Growth Arrest and DNA Damage Inducible Gene	(GADD53)
Hemagglutinin	(HA)
Hormone Response Unit	(HRU)
HEPES-Buffered Saline Buffer	(HBS)
Kilobase	(kb)

Kilodalton	(kDa)
Isopropyl-β-D-thiogalactopyranoside	(IPTG)
Liver-Enriched Transcriptional Activator Protein	(LAP)
Liver-Enriched Transcriptional Inhibitor Protein	(LIP)
Liver Specific Region	(LSR)
Low Melting Point	(LMP)
Luria-Bertani Ampicillin	(LBA)
Messenger RNA	(mRNA)
Melting Temperature	(T_m)
N,N,N′,N′-Tetramethylenediamine	(TEMED)
N-2-Hydroethylpeperazine-N′-2-Ethane Sulphonic Acid	(HEPES)
o-Nitrophenyl-β-D-Galactopyranoside	(ONPG)
Polyacrylamide Gel Electrophoresis	(PAGE)
Phosphate Buffered Saline	(PBS)
Phosphate Buffered Saline Tween	(PBST)
Phosphoenolpyruvate	(PEP)
Phosphoenolpyruvate carboxykinase	(PEPCK)
Phenylmethanesulfonyl Fluoride	(PMSF)
Polyethylene Glycol	(PEG)
Polymerase Chain Reaction	(PCR)
Protein Kinase A	(PKA)
Protein Kinase C	(PKC)
Polyvinylidene Fluoride	(PVDF)
Radio-Immuno-Precipitation Assay Buffer	(RIPA)
Ribonucleic Acid	(RNA)
Standard Error	(S.E.)
Sodium Chloride	(NaCl)
Sodium Dodecyl Sulphate	(SDS)
Sodium Hydroxide	(NaOH)
TATA Binding Protein	(TBP)
TATA binding Protein Associated Factors II B	(TFIIB)
TATA binding Protein Associated Factors II F	(TFIIF)
Terrific Broth	(TB)
Terrific Broth Ampicillin	(TBA)
Transactivation Domain	(TA)
Triple Mutant	(TM)
Tris-[hydroxymethyl]-aminomethane	(Tris)
Tris-Acetate EDTA Buffer	(TAE)
Tris-EDTA Buffer	(TE)

1. INTRODUCTION AND OVERVIEW

Phosphoenolpyruvate carboxykinase (PEPCK) gene is a paradigm for the study of transcription regulation. The PEPCK enzyme has no known allosteric regulators, it is not post-translationally modified, the gene/protein has tissue specific and developmentally specific patterns of expression and its expression is regulated almost entirely at the level of transcription initiation. Because PEPCK is a key regulator of gluconeogenesis, its gene promoter is composed of a complex array of *cis*-elements that allow its rate of expression to be responsive to a variety of hormones.

Studies performed on the PEPCK gene promoter have revealed that its cyclic AMP (cAMP) response unit consists of three binding sites for CCAAT/enhancer binding protein α (C/EBPα) and/or C/EBPβ, as well as an Activator Protein-1 (AP-1) site, and a cAMP Response Element (CRE) site that can bind C/EBPα, C/EBPβ or cAMP Response Element Binding Protein (CREB) (Park *et al.*, 1990; Roesler *et al.*, 1994, 1998; Roesler, 2000; Short *et al.*, 1986). C/EBPα and C/EBPβ are liver enriched transcription

factors that participate in the protein kinase A (PKA)-inducible response of PEPCK and a number of metabolically important genes. The goal of this thesis is to determine if these isoforms share any regions of homology between their transactivation domains and, if so, to determine whether the conserved regions mediate the PKA-inducible activity when they bind to the PEPCK promoter. As the results section of this thesis will show, the homologous domains within C/EBPα, but not C/EBPβ, are critical for mediating the PKA-inducible response on the PEPCK promoter. These data suggest that although these isoforms share regions of conserved amino acids, these regions may not share a conserved function. Also, this thesis will indicate that promoter environment may influence which domains within C/EBPα mediate the constitutive and PKA-inducible activities on the PEPCK promoter.

To gain a greater understanding for how this research project is designed, the literature review is outlined as follows: Section 2.1 describes the basic concepts of transcriptional regulation and this section expands into the study of hormonal regulation of a gene promoter in greater detail. Section 2.2 evaluates the hormonal regulation of the PEPCK gene promoter. Section 2.3 describes the C/EBP protein family in detail in terms of their biological and metabolic roles, and their structure and

function. Section 2.4 describes the transactivational activity of C/EBPα and C/EBPβ. Finally, section 2.5 outlines the specific objectives of this thesis.

2. REVIEW OF THE LITERATURE

2.1 Overview of Transcription Regulation

Eukaryotic gene expression is a two-stage process that is functionally and physically separated in the cell. Transcription, the synthesis of messenger RNA (mRNA) from the deoxyribonucleic acid (DNA) template, is localized to the nucleus whereas translation, the synthesis of proteins from the mRNA template, is localized to the cytoplasm. Transcriptional regulation is central to tissue specific gene expression and cellular response to a specific stimulus (Latchman, 1997). The major point of regulation of gene expression is transcription initiation although mRNA degradation and post-transcriptional modifications do contribute to the control of gene expression. Most eukaryotic genes are expressed at a constitutive level until their rate of transcription is induced in response to a stimulus (Barberis and Gaudreau, 1998).

The regulatory structure of a eukaryotic gene consists of a proximal upstream promoter and distant enhancer sequences (Beyersmann, 2000). An ordered assembly of general transcription factors combine to form the

preinitiation complex, including Transcription Factor Class II Gene F (TFIIF) which escorts RNA polymerase II to the promoter to initiate transcription (Latchman, 1997; Smale, 2001). Enhancers are found at variable distances from the proximal promoter, upstream or downstream of the initiation start site and they often mediate an effect independent of orientation. An enhancer consists of *cis*-elements or specific sites on the DNA template that can be bound by specific and often inducible trans-activation factors (Figure 2.1). The ability of these proteins to induce or repress gene expression may be altered by post-translational modification and the presence or absence of accessory factors (Beyersmann, 2000, Chang and Jaehning, 1997).

A protein is constitutively expressed when the general transcription factors form the pre-initiation complex and recruit RNA polymerase II to the promoter. Hormonal induction of transcription requires the correct assembly of the preinitiation complex as well as the correct assembly of inducible transcription factors tethered to sites within the enhancer region. The number and identity of *cis*-elements on a promoter varies from gene to gene and the identity of transcription factors that bind to each *cis*-element may vary depending on the regulatory

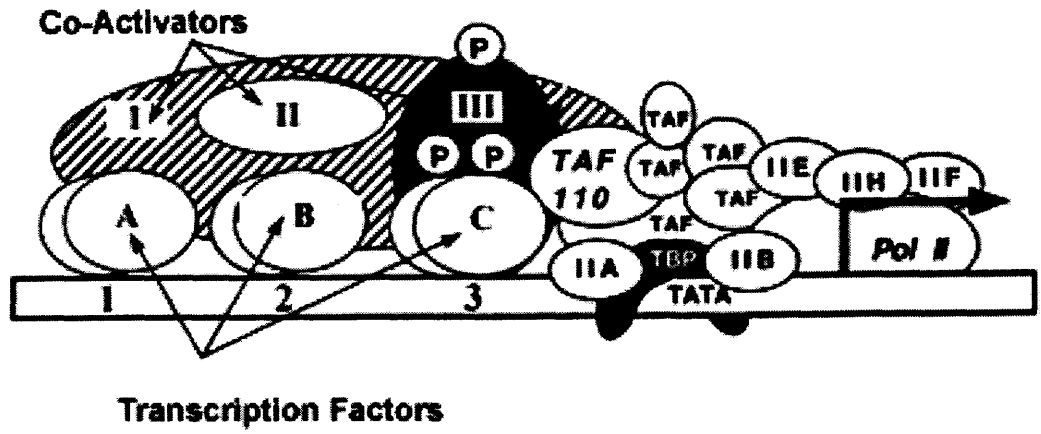


Figure 2.1: Schematic diagram of a hypothetical TATA-containing promoter. This diagram identifies general transcription factors (including the TATA binding protein (TBP) and its associated TATA-binding protein associated factors (TAFs), various transcription factors and co-activators. The general transcription factors and RNA polymerase II associate to form the preinitiation complex. Upstream transcription factors (A, B and C) bind to DNA *cis*-elements (1, 2 and 3). These transcription factors may be phosphorylated and associate with various co-activators (I, II, III) which may act as a bridge between the enhancer region and the general transcription factors.

properties of the gene and which hormones are present (for reviews see Lucas and Granner, 1992 and Roesler and Park, 1998).

Transcription factors are often modular proteins with a distinct domain for tethering the protein to a specific *cis*-element on the DNA and a transactivation domain for mediating the stimulatory or inhibitory effect on gene expression (Latchmann, 1997). While not all transcription factors bind to consensus sites on the DNA, this is generally the case. The most common DNA binding domain motifs are the helix-loop-helix motif, the basic region-leucine zipper domain (bZIP), and the zinc finger domain (Beyersmann, 2000; Latchman, 1997; Mitchell and Tijan, 1989). Transactivation domains are characterized as glutamine rich, acidic amino acid rich or proline rich and these domains mediate the protein-protein interaction between transcription factors, co-activators and/or members of the preinitiation complex (Beyersmann 2000; Chen, 1999; Mitchell and Tijan, 1989). The actions of DNA binding domains and transactivation domains are often physically and functionally distinct from each other, which means that the DNA binding domain of a transcription factor can be replaced with an alternate DNA binding domain, often with no discernible effect on the activity of the transactivation domain.

2.1.1 Hormone Response Units

The presence of hormones can lead to the alteration of the expression of countless genes, especially genes that code for proteins that form the rate-limiting enzymes of metabolic pathways (Lucas and Granner, 1992). It has recently been elucidated that for a hormone to affect gene transcription, the gene promoter may be composed of several *cis*-elements that synergize to mediate a robust response. These *cis*-elements are collectively referred to as a hormone response unit (HRU) (reviewed in Roesler and Park, 1998). Each HRU is composed of a hormone response element which mediates a measurable response to the hormone in isolation but it cannot account for the full effect of the hormone on gene expression. The HRU may be composed of one or more accessory elements which bind accessory factors. Accessory factors are transcription factors which fail to mediate a measurable hormonal response independently but they serve to amplify the response of the transcription factor that binds the hormone response element. An accessory factor may serve to stabilize protein-DNA interactions at the hormone response element, it may stabilize protein-protein interactions between the hormone response element and the general transcription factors and it may also assist in recruitment of co-activators to the

promoter (Roesler and Park, 1998). Each hormone that acts upon a specific gene promoter may have its own HRU which may include unique and/or shared *cis*-elements. Multiple transcription factors may bind to a single site within the HRU and the unique combination of transcription factors allow for a "fine-tuned" response of the promoter to the hormone. Essentially, a gene can exist in altered states of hormonal responsiveness depending on the particular combination of transcription factors that occupy specific *cis*-elements within the HRU (Roesler, 2000).

2.2 Overview of PEPCK Gene Expression

The PEPCK enzyme is a key regulator of gluconeogenesis in the liver, and gluconeogenesis and ammoniagenesis in renal cells (for reviews see Hanson and Patel, 1994 and Hanson and Reshef, 1997). However, PEPCK is also expressed in non-gluconeogenic tissues such as adipose tissue, lactating mammary glands and the small intestine (Anderson, 1970; Ballard, *et al.*, 1967; Feldman and Hirst, 1978; Lobato, *et al.*, 1985; Zimmer and Magnuson, 1990). In non-gluconeogenic tissues, PEPCK mediates limited glyceroneogenesis during starvation by producing 3-phosphoglycerate which is used to convert free fatty acids into di- or tri-acylglycerols. PEPCK catalyzes the conversion of oxaloacetate to

phosphoenolpyruvate and carbon dioxide (CO_2) using GTP as a substrate and releasing GDP as a byproduct.

PEPCK is the first committed step in gluconeogenesis in liver and kidney cells and its expression is induced during the perinatal period concomitant with an increased need for self-sufficient gluconeogenesis (Hanson and Reshef, 1997; Rognstad, *et al.*, 1979). There are cytosolic and mitochondrial isoforms of PEPCK which are encoded by separate genes but the mitochondrial isoform is not induced by any known stimuli and will therefore not be discussed in this thesis. The expression of cytosolic PEPCK is altered in the liver by dietary and hormonal stimuli. Its expression is up-regulated during starvation, after prolonged exercise, in the presence of increased levels of glucocorticoids and in the diabetic state; however, its expression is reduced in response to insulin and after a prolonged, high carbohydrate diet (Alleyne and Scullard, 1969; Friedman, 1994; Hanson and Patel, 1984; Lamers, *et al.*, 1982; Sasaki, *et al.*, 1984, Tilghman, *et al.*, 1974).

2.2.1 Transcriptional Regulation of the PEPCK Gene in Liver

Although PEPCK is a key regulatory enzyme in gluconeogenesis, it is not post-translationally modified, it has no known allosteric modifiers, and its expression is regulated almost entirely at the level of transcription. However, there is limited control of PEPCK's mRNA stability. Its mRNA half-life is increased in response to fasting and it is extended from approximately 30 minutes to nearly four hours in the presence of cAMP (Hod, *et al.*, 1988; Nachaliel, *et al.*, 1993; Nelson, *et al.*, 1980). By increasing PEPCK's mRNA half-life, PEPCK expression can be induced during times of insufficient glucose uptake.

PEPCK expression is induced in the presence of cAMP, glucocorticoids, retinoic acid and thyroid hormone and its expression is inhibited by insulin (Granner, *et al.*, 1983; Lamers, *et al.*, 1982; Loose, *et al.*, 1985; Lucas, *et al.*, 1991). By utilizing multiple HRUs to mediate these responses, the PEPCK promoter has increased control over its response to hormones and tissue specific and developmental cues. The effects of glucagon (via cAMP) and the cAMP response unit will be discussed in detail in the following section.

2.2.1.1 The cAMP Response Unit

It is only recently that the mechanism whereby cAMP activates gene expression in eukaryotes has expanded from the limited view that a CRE mediates the response to the much more complex view that a HRU mediates the cAMP response. The CRE is a palindromic sequence with the consensus sequence 5'TGACGTCA3' and it is the binding site for the transcription factor CREB (Hoeffler, *et al.*, 1988; Montminy and Bilezikjian, 1987; Shaywitz and Greenberg, 1999). Initially, it was thought that CREB alone mediated the cAMP response. CREB is phosphorylated on a single serine residue by PKA and this phosphorylation "activates" CREB (Shaywitz and Greenberg, 1999). Upon phosphorylation, CREB can bind CREB Binding Protein, a co-activator that induces gene expression by contacting several members of the preinitiation complex and by altering the promoter environment with its intrinsic histone acetylase activity (Chrivia, *et al.*, 1993, Lee, *et al.*, 2001). This limited CRE model has several failings. It does not account for the tissue specificity that some cAMP responsive genes display because CREB and CREB binding protein are ubiquitously expressed. Furthermore, it does not explain how a CRE site may fail to mediate a strong cAMP response in some genes. The PEPCK gene is a perfect example to demonstrate these limitations. PEPCK is

robustly responsive to cAMP in the liver but it mediates a limited cAMP response in the kidney (Lamers, *et al.*, 1982; Meisner, *et al.*, 1985). Transfection analysis with a PEPCK promoter that is truncated after the CRE site is only weakly responsive in the presence of PKA compared to the PEPCK promoter that extends out to nucleotide –490 (Roelser, *et al.*, 1993). Obviously, ubiquitiously expressed CREB cannot account for PEPCK's tissue specific PKA-inducible response; other proteins upstream of the CRE are required to mediate the PKA-inducible effect. These observations led to the discovery and elucidation of a cAMP response unit (CRU) within the PEPCK promoter that is responsible for mediating this hormonal response.

In an attempt to identify transcription factors that may help mediate the PKA-inducible response, DNase I footprinting of the PEPCK promoter was performed using nuclear extracts from rat liver, spleen, kidney and brain tissues (Roesler, *et al.*, 1989). In conjunction with a footprint at the nonconsenesus CRE site within the PEPCK promoter, a distinct liver-specific footprinting pattern was identified between –230 and –320 bp. As expected, the CRE site could be occupied by recombinant CREB but competition with an oligonucleotide which contains a CRE sequence was noticed to compete with the protein at the CRE and an

upstream region. Transient transfection analysis identified that a robust response to PKA was only realized if the upstream region was included with the CRE on the promoter (Roesler, *et al.*, 1993). The importance of the upstream site for determining the tissue-specific response was elucidated when it was identified that the liver specific transcription factor, C/EBPα bound to three specific sites within the PEPCK promoter (Park, *et al.*, 1990; Roesler, *et al.*, 1996). Extensive analysis of the various *cis*-elements on the PEPCK promoter identified an upstream region (now termed the Liver Specific Region (LSR)) which consists of an AP-1 binding site and three C/EBP binding sites. Together with the CRE site, these *cis*-elements constitute the CRU (Figure 2.2) (Roesler, *et al.*, 1994). There appears to be no specific architectural requirement for these five *cis*-elements but the three C/EBP binding sites, the AP-1 site and the CRE must all be present for a robust PKA-inducible response to be realized (Liu, *et al.*, 1991; Roesler, *et al.*, 1994).

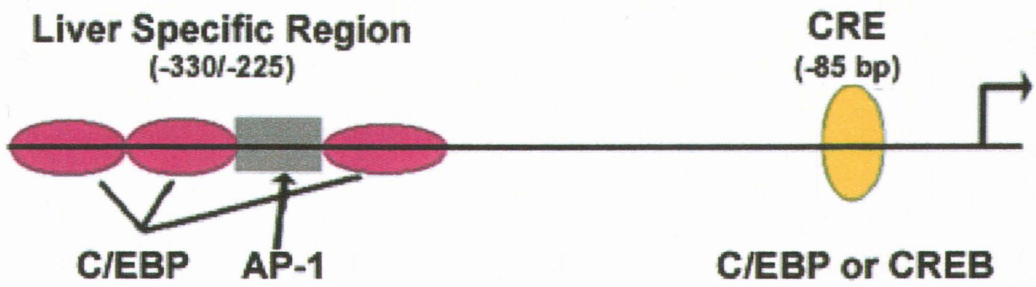


Figure 2.2: Schematic of the cAMP response unit of the PEPCK promoter. The CRU of the PEPCK promoter is composed of five *cis*-elements; three C/EBP binding sites and an AP-1 binding site within the Liver Specific Region (-330/-225 bp) and a CRE at position -85 bp from the initiation start site.

Using multiple experimental techniques including recombinant protein expression, transient transfection analysis and mutation analysis (*i.e.* converting the nonconsensus CRE site within the PEPCK promoter to a consensus C/EBP site or CRE site,) it was determined that the CRE element in the PEPCK promoter can be occupied by C/EBPα, C/EBPβ and CREB and binding of any of these three transcription factors (within the context of the CRU) results in a strong PKA-inducible response (Liu, *et al.*, 1991; Park, *et al.*, 1990; 1993; Roesler, *et al.*, 1989; 1995; 1998; Wilson, *et. al.*, submitted). Previously, it was believed that C/EBPβ bound to this site to mediate the glucocorticoid response but binding to the CRE inhibited the cAMP response (Roesler, *et al.*, 1998; Yamada, *et al.*, 1999). Further transient transfection analysis showed that a truncated version of C/EBPβ could mediate a strong PKA-inducible response when it bound to the CRE (Wilson, *et. al.*, submitted)

Using GAL4 methodology, a robust PKA-inducible response could be observed when C/EBPα or truncated C/EBPβ was tethered to the distal sites on the PEPCK promoter (Park, *et al.*, 1999; Roesler, *et al.*, 1996). Briefly, this methodology entails removing the endogenous DNA binding domain of a specific transcription factor and replacing it with the DNA binding domain of the yeast transcription factor, GAL4. This recombinant

protein was co-expressed in the presence of a permissive PEPCK promoter which had specific sites within the CRU replaced with one or more GAL4 site. It was discovered that either C/EBPα or C/EBPβ could potentially occupy the three sites within the LSR to mediate a vigorous PKA-inducible response irrespective of which of the three proteins occupied the CRE (Park, *et al.*, 1999; Roesler, *et al.*, 1996). A limitation of the GAL4 methodology is that it only indicates a protein's potential to mediate a hormonal response on a reporter gene but it does not address the effect of each protein on the endogenous promoter *in vivo* (Fry and Farnham, 1999). Crosson and Roesler, (2000) addressed these limitations by inhibiting the expression of specific C/EBP isoforms using antisense methodology and studying the effect on the cAMP responsiveness of the PEPCK promoter. Using H4IIE cells, a liver cell line which expresses PEPCK and responds to hormones in a manner analogous to hepatocytes, they identified that C/EBPα, but not C/EBPβ, is absolutely required to mediate the cAMP response on the PEPCK promoter (Crosson and Roesler, 2000).

How each transcription factor competes for binding to the distinct sites within the CRU is not fully understood. It is speculated that the relative abundance of each transcription factor may lead to increased competition for sites on the DNA. Because of the high degree of amino

acid similarity shared between the bZIP domains of C/EBPα and C/EBPβ, the possibility of C/EBPα/β heterodimers occupying sites in the CRU must also be considered.

2.3 The CCAAT/Enhancer-Binding Protein Family of Transcription Factors

C/EBPα was purified from rat liver nuclear extracts and characterized as a heat-stable, DNA binding protein (Graves, *et al.*, 1986; Johnson, *et al.*, 1987). Upon discovery of this protein, it became evident that C/EBPα marked a new class of transcription factors that interact with their DNA targets via a basic region-leucine zipper (bZIP) region. This DNA binding protein recognizes the DNA sequence 5'ATTGCGCAAT3' but it can bind *cis*-elements that vary considerably from this consensus sequence (Agre, *et al.*, 1989; Osada, *et al.*, 1996). Eight isoforms from six genes compose the C/EBP transcription factor family. All family members share significant structural similarities in the bZIP domain although they diverge in their tissue specificity, transactivational activity and developmental expression patterns.

Many researchers have cloned the C/EBPβ gene from several animal species leading to different nomenclature and confusion in the

literature. For the sake of clarity, C/EBPβ will be used to represent all the animal species unless otherwise indicated.

2.3.1 Biological Roles of C/EBP Isoforms

C/EBP transcription factors are critical for normal cellular differentiation, and developmental and tissue specific gene expression (Lekstrom-Himes and Xanthopoulos, 1998). C/EBPα is an intronless gene that is expressed at high levels in the liver, lung, white adipose tissue, brown adipose tissue and the placenta with its expression being the highest in the liver and adipose tissue (Birkenmeir, *et al.*, 1989). C/EBPα plays a significant role in mediating the expression of metabolically important genes in the liver, in fetal lung development and in initiating hepatocyte cell cycle arrest (Birkenmeir, *et al.*, 1989; Darlington, 1999; Flodby, *et al.*, 1996). Darlington and colleagues have investigated the role of C/EBPα in the acute phase response using C/EBPα knockout mice. Northern blot analysis indicated that an inflammatory stimulus failed to induce the acute phase response in C/EBPα knockout mice, and that mice without the C/EBPα gene displayed an early block in the maturation of granulocytes (Zhang, *et al.*, 1997). This study is one of the first to indicate that C/EBPα has a role in the acute phase response.

Constitutive expression of C/EBPβ is highest in the liver, intestine, adipose tissue and lung (Cao, *et al.*, 1991). C/EBPβ was originally identified as a master regulator of the acute phase response (Akira, *et al.*, 1990). Like C/EBPα, C/EBPβ also lacks introns. Akira and colleagues identified C/EBPβ as a nuclear factor that can occupy the interleukin-1 element on the interleukin-6 promoter and induce its expression (Akira, *et al.*, 1990). Further analysis indicated that C/EBPβ can induce the expression of a number of genes in the liver such as tumor necrosis factor, interleukin-8, and granulocyte colony-stimulating factor, genes that are vital for the acute-phase reaction, inflammation and hematopoiesis (Akira, *et al.*, 1990). C/EBPβ knockout mice have altered T-helper cells and humoral immunity, they are more susceptible to skin lesions, they have elevated interleukin-6 in their blood and they developed splenomegaly and lymphoadenopathy (Screpanti, *et al.*, 1995; Tanaka, *et al.*, 1995). Clearly, C/EBPβ plays a major role in regulating the immune response.

After preadipocyte cells have been induced to differentiate into adipose cells, there is a significant, albeit transient, increase in the expression of C/EBPβ and C/EBPδ which may initiate adipogenesis by inducing the expression of C/EBPα (Cao, *et al.*, 1991). Ectopic expression of C/EBPβ promotes adipogenesis in pre-adipocytes and fibroblast cell

lines and knockout models of C/EBPβ and C/EBPδ result in pre-adipocytes that fail to differentiate despite the presence of the appropriate stimuli (Tanaka, *et al.*, 1997; Wu, *et al.*, 1995). Studies show that C/EBPβ also plays a role in epithelial cell proliferation, mammary gland differentiation and C/EBPβ knockout mice females are sterile (Robinson, *et al.*, 1998; Sterneck *et al.*, 1997).

C/EBPδ is an intronless gene which is expressed in intestine, adipose tissue and lung, and its expression is stimulated in all tissues after lipopolysaccharide stimulation (Cao, *et al.*, 1991; Yin, *et al.*, 1996). C/EBPδ and C/EBPβ are expressed in the early stages of adipocyte differentiation and they have a role in inducing the expression of C/EBPα and other adipocyte marker genes (Lane, *et al.*, 1999; Tanaka, *et al.*, 1997). C/EBPα, β and δ are emerging as regulators of the cAMP response in a number of genes revealing the importance of this class of transcription factors in regulating hormonal responsiveness (Wilson and Roesler, 2002).

C/EBPγ is a 16 kD protein which is ubiquitously expressed with its highest level of expression in progenitor cells. This protein can heterodimerize with other family members; however, because it lacks a transactivation domain, dimerization with other family members creates inactive heterodimers. (Cooper, *et al.*, 1995).

The C/EBPε gene consists of two introns and five in-frame translation initiation sites. The protein is expressed primarily in myeloid and lymphoid cells and this gene can be transcribed into four mRNA species (Chumakov, *et al.*, 1997). These mRNA isoforms are translated to produce proteins with identical leucine zipper domains but different amounts of transactivation domain (Tang and Koeffler, 2001).

C/EBPζ (also known as growth arrest and DNA damage inducible gene (gadd53)), is a ubiquitously expressed protein which readily heterodimerizes with other C/EBP isoforms. Dimerization creates inactive heterodimers due to the presence of two proline residues within the DNA binding region of C/EBPζ. These proline residues make it impossible to bind DNA, which results in C/EBPζ functioning as a dominant negative inhibitor of C/EBP transcriptional activity (Ron and Habener, 1992).

2.3.1.1 Metabolic Roles of C/EBPα

In 1989, McKnight and colleagues posed the question, "Is CCAAT/enhancer-binding protein a central regulator of energy metabolism?" based on the observation that its highest levels of expression was found in tissues such as liver, adipose and lung, which are organs that control energy metabolism and/or actively metabolize lipids

(Birkenmeier, *et al.*, 1989, McKnight, *et al.*, 1989). The work by Darlington and colleagues has been instrumental in defining C/EBPα as a regulator of adipose tissue development (Darlington, *et al.*, 1998; Wang, *et al.*, 1995). They used homologous recombination and cross-breeding experiments to create mice with a deletion of the C/EBPα gene (C/EBPα-/-) (Wang, *et al.*, 1995). At birth, these mice had no gross abnormalities. Their blood glucose levels were normal, their organs appeared normal, their weight was normal and the C/EBPα-/- mice could not be distinguished from their littermates. However, several hours after birth, the C/EBPα-/- mice became increasingly lethargic and most died within 8 hours. Blood glucose levels taken 5-8 hours post-partum showed a significant drop compared to the homozygous normal and heterozygous littermates. These profoundly hypoglycemic mice could be temporarily rescued if they were injected with a glucose solution every 7 hours after birth.

Pathology showed that, unlike the control littermates, the C/EBPα -/- mice had very little liver glycogen prenatally and no liver glycogen postnatally. These mutant mice had significantly reduced lipid droplets in their adipose tissue and Northern analysis showed a significantly reduced expression of uncoupling protein in brown adipose tissue (Wang, *et al.*, 1995). The C/EBPα-/- mice had significantly reduced hepatic mRNA

levels of serum albumin, glycogen synthase, PEPCK and glucose-6-phosphatase relative to control mice in the perinatal period and they had immature lungs. It was observed, however, that after 32 hours, the mRNA levels of PEPCK and glycogen synthase recovered suggesting that another C/EBP isoform may be partially compensating for the loss of C/EBPα.

Because the C/EBPα knockout mice had a <1% survival rate after the perinatal period, Darlington and colleagues designed a variant of the C/EBPα-/- mice which expressed C/EBPα in hepatocyte tissue exclusively (Linhart, *et al.*, 2001). These C/EBPα -/- mice were designed with the region coding for the C/EBPα protein under the direct transcriptional control of the serum albumin enhancer region and proximal promoter, a gene which is expressed exclusively in the liver (albumin$^{\alpha/\alpha}$/ C/EBPα-/- mice). Northern blot analysis confirmed that C/EBPα was expressed in liver cells but not in any other tissues and this new knockout model had a 3-fold better survival rate than the original C/EBPα-/- mice. The mRNA expression of PEPCK, glycogen synthase and bilirubin UDP-glucuronosyltransferase were normal in the albumin$^{\alpha/\alpha}$/ C/EBPα-/- mice, however, these mice showed a selective absence of white adipose tissue. Lipid accumulation in brown adipose tissue was delayed but by day 7, it

was readily detectable and had minimal biochemical alterations. Mammary gland fat tissue was morphologically normal.

Lee and colleagues also recognized the limitations of the first C/EBPα-/- model which only allowed the study of C/EBPα expression in the neonate (Lee, *et al.*, 1997a). To gain greater insight into the role of C/EBPα in the adult mouse, they constructed a conditional knockout allele of the C/EBPα gene using the Cre/LoxP recombinase system to study the effects of altered C/EBPα expression specifically in the liver of the adult mouse (Lee, *et al.*, 1997a). These mice were phenotypically normal compared to their control littermates until Cre recombinase was delivered to the hepatocytes via infusion of a recombinant adenovirus. Studies showed that, upon infection, more than 80% of the C/EBPα gene was deleted in the liver and the expression of C/EBPα protein was reduced by 90%. These conditional knockout mice had significantly reduced bilirubin UDP-glucuronosyltransferase expression in the liver which led to the mice developing severe jaundice. Similar to the perinatal knockout mice, the expression of PEPCK and glycogen synthase was significantly reduced, as was factor IX. These data suggest that not only is C/EBPα important in the normal expression of PEPCK and glycogen synthase in the neonate, it

is important for maintaining the expression of these enzymes in the adult liver.

Adipogenesis is controlled by a complex array of transcription factors like C/EBPα, β, δ and peroxisome proliferator activated protein γ which are expressed at different stages of the differentiation process (Darlington, *et al.*, 1995; 1998). C/EBPα inhibits cellular proliferation and increases the expression of adipose marker genes which leads to the adipocyte phenotype (Darlington, *et al.*, 1995; 1998; Lane, *et al.*, 1999). Many experiments emphasize that although C/EBPα is expressed relatively late in the differentiation process, C/EBPα is a master regulator of adipocyte differentiation. When C/EBPα was over-expressed in 3T3-L1 preadipocyte cells, the differentiation process was initiated despite the lack of any hormonal signal (Lin and Lane, 1992). Ectopic expression of C/EBPα in a preadipocyte cell line not only conferred the adipocyte phenotype but it also conferred insulin-responsive glucose uptake (El-Jack, *et al.*, 1999). When C/EBPα antisense mRNA was introduced into the same cell line, preadipocyte differentiation was inhibited but subsequent introduction of sense C/EBPα mRNA overcame this inhibition (Lin and Lane, 1992). 3T3-L1 preadipocyte cells transfected with the C/EBPα gene showed inhibited adipoblast growth compared to the control cells but

within 5 days the cells developed the adipocyte morphology (Freytag and Geddes, 1992).

Karagiannides and colleagues (2001) studied the effects of altered C/EBP protein expression in aging mice. Northern blot analysis indicated that C/EBPα levels in adipocytes declined with age and over-expression of C/EBPα into preadipocytes cultured from old rats restored the differentiated adipocyte morphology and fat cell storage capacity (Karagiannides, *et al.*, 2001). The overall expression level of C/EBPβ did not alter with age in mice but a truncated C/EBPβ isoform did increase in cultured preadipoctyes and isolated fat cells of aged mice. These data indicate that alterations in C/EBPα and C/EBPβ expression may contribute to age-related impaired adipogenesis and fat tissue function.

Many adipose specific genes such as the *ob* gene (which codes for leptin) and the *aP2* gene (which codes for a lipid binding protein) have consensus sites for C/EBPα in their proximal promoters which suggests that their expression is under the direct control of C/EBPα (Lane, *et al.*, 1999; Herrera, *et al.*, 1989; Hollenberg, *et al.*, 1997; Hwang, *et al.*, 1996). The promoter region of the C/EBPα gene has a C/EBP binding site which can be occupied by C/EBPβ and C/EBPδ. Once activated, C/EBPα can

stimulate its own production to maintain the terminally differentiated state (Christy, *et al.*, 1991; Timchenko, *et al.*, 1995).

2.3.1.2 Metabolic Roles of C/EBPβ

C/EBPβ was first identified as a key element of interleukin signaling and induction of a number of acute phase response genes (Akira, *et al.*, 1990; Poli, *et al.*, 1990). To study the effects of C/EBPβ deficient mice on interleukin-6 expression, Tanaka and colleagues generated a null mutant of the C/EBPβ gene by gene targeting (Tanaka, *et al.*, 1995). They observed that C/EBPβ-/- mice were prone to infection and they had induced expression of cytokines but, overall, these mice appeared phenotypically normal (Tanaka, *et al.*, 1995). However, Tanaka and others identified that the Mendelian ratio of mice heterozygous for the C/EBPβ deletion was significantly lower than expected from inheritance and they suggested that this was due to a number of mice dying *in utero* (Screpanti, *et al.*, 1995; Tanaka, *et al.*, 1995).

Researchers have studied C/EBPβ knockout models and a complex pattern of gene expression has been elucidated. Although there are multiple phenotypes observed with C/EBPβ knockout mice, only the B phenotype and the A phenotype will be discussed in this thesis. Fifty

percent of the C/EBPβ-/- mice have the B phenotype and these mice die shortly after birth due to their inability to mobilize glycogen stores and to express hepatic PEPCK (Croniger, *et al.*, 1997). Administration of cAMP results in the mobilization of liver glycogen, induced PEPCK expression and the normalization of blood-glucose levels (Liu, *et al.*, 1999). Liu and colleagues determined that the surviving C/EBPβ-/- mice had fasting hypoglycemia and decreased blood lipids. They determined that C/EBPβ was not required for the normal induction of PEPCK, glucose-6-phosphatase or glucokinase in response to hormone stimulation directly but C/EBPβ was required to ensure the appropriate concentrations of cAMP which directly effect lipid and glucose homeostasis (Liu, *et al.*, 1999). This latter observation was confirmed by Croniger, *et al.*, (2001) who identified that C/EBPβ-/- mice have 50% less hepatic cAMP compared to control mice due to a decreased concentration of PKA and an increased concentration of cAMP phosphodiesterases (Croniger, *et al.*, 1998; 2001).

The C/EBPβ-/- A phenotype mice had normal glucose homeostasis but these mice died after 4-6 months due to problems related with a severely compromised immune system (Croniger, *et al.*, 1997; Screpanti, *et al.*, 1995; Tanaka, *et al.*, 1995). The A phenotype mice had significantly

reduced lipid accumulation, the females were sterile, they had difficulty in maintaining fasting blood glucose homeostasis, and they had altered epithelial cell proliferation, and mammary gland differentiation (Croniger, *et al.*, 1997; 2001; Liu, *et al.*, 1999; Robinson *et al.*, 1998; Screpanti, *et al.*, 1995; Sterneck, *et al.*, 1997; Tanaka, *et al.*, 1997). Pathology of these mice showed that they had high circulating blood levels of interleukin-6, they had altered T-helper and humoral immunity elevating their susceptibility to infection (Cao, *et al.*, 1991; Screpanti *et al.*, 1995; Tanaka, *et al.*, 1995).

A fascinating study was undertaken by Chen and colleagues in which the Cre/LoxP method was used to replace the entire protein-coding region of C/EBPα with C/EBPβ within the context of the C/EBPα gene locus (C/EBP$\alpha^{\beta/\beta}$) (Chen, *et al.*, 2000). C/EBP$\alpha^{\beta/\beta}$ mice were viable, their gross morphology was normal and they had similar body weight compared to their wild-type littermates. Unlike the previous knockout models of C/EBPα, these mice had no liver dysfunction, they had normal blood glucose and albumin levels and both sexes were fertile (Chen, *et al.*, 2000; Darlington, *et al.*, 1999; Lee, *et al.*, 1997). Northern blot analysis identified that the concomitant gain of C/EBPβ expression under control of the C/EBPα gene locus in the C/EBP$\alpha^{\beta/\beta}$ mice did not differ from the C/EBPα expression patterns of the wild-type litter mates which confirmed

that the genetic manipulation did not affect gene expression. Also, the expression level of endogenous C/EBPβ in C/EBPα$^{\beta/\beta}$ and wild-type littermates was unaffected. Northern blot analysis of C/EBPα$^{\beta/\beta}$ mice showed that the PEPCK mRNA levels and timing of expression was similar to that of wild-type mice.

A closer examination of the C/EBPα$^{\beta/\beta}$ mice indicated that although these mice had similar body weights compared to their wild-type littermates, their white adipose tissue was small and yellowish in colour, unlike the heterozygous and wild-type littermates whose white adipose tissue was large and white. The lipid accumulation in these mutant mice was inhibited although the C/EBPα$^{\beta/\beta}$ mice had 60% more brown adipose tissue than the other littermates. Northern analysis showed that genes involved in adipogenesis in white adipose tissue of C/EBPα$^{\beta/\beta}$ mice were not affected by the loss of C/EBPα expression; however, the mRNA and protein levels of leptin and adipsin, both of which are white adipose tissue marker genes, were significantly reduced in these mice. Unlike the binding pattern of C/EBPβ in hepatocytes, C/EBPβ has been shown to bind to putative distal elements in the PEPCK promoter and transactivate its expression in adipocytes (Eubank, *et al.*, 2001). C/EBPβ can functionally

replace C/EBPα in the liver but not in adipose tissue when it is expressed from the C/EBPα gene locus.

2.3.2 Structure/Function Analysis of C/EBPα and C/EBPβ

C/EBP proteins are modular transcription factors that are composed of an N terminal transactivation domain and a carboxy terminal DNA binding domain (Figure 2.3). C/EBPα and C/EBPβ are extremely G-C rich, intronless genes with the corresponding amino acids in their DNA binding domains sharing approximately 71% identity (Trautwein, *et al.*, 1995). However, the amino acid sequence between their transactivation domains, the region of the proteins that mediate the constitutive and inducible transactivation potential of the transcription factor, are largely unique.

2.3.2.1 The DNA Binding Domain of C/EBP

The bZIP domain of C/EBP proteins consists of a bipartite motif that is responsible for both dimerization and DNA *cis*-element recognition and binding (Landschulz, *et al.*, 1989). The leucine zipper domain is an approximately 30 amino acid segment of the C/EBP protein that resides

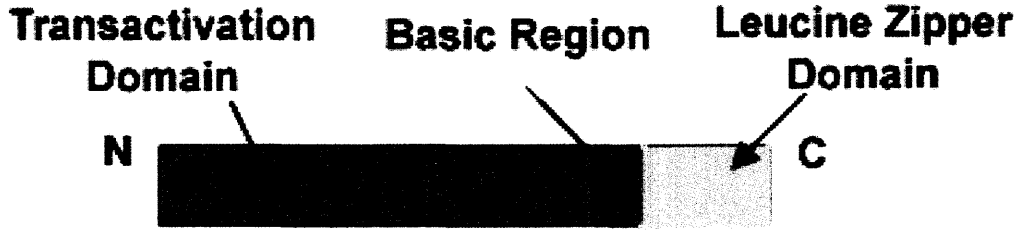


Figure 2.3: Schematic diagram of C/EBP proteins. The transactivation domain resides at the N terminus, the leucine zipper resides at the C terminus and the basic region is immediately N terminus to the leucine zipper motif.

at the extreme C terminus of the protein. The carboxy terminal region of C/EBP proteins contains 5 leucines repeated at every seventh amino acid that corresponds to every two turns of the α-helix if the α-helix over-twists slightly (Krylov, *et al.*, 1994). Using a helical wheel analysis, the α-helix displays considerable amphipathy: one side of the helix is composed of a heptad repeat consisting of hydrophobic amino acids and leucines at positions 1 and 4 in the repeat, and the other side of the wheel has a significant number of charged amino acids (6 basic amino acids and 4 acidic amino acids) and uncharged, polar amino acids (Figure 2.4) (Krylov, *et al.*, 1994). The basic region of the C/EBP protein is composed of a short stretch of basic amino acids immediately N-terminal to the leucine zipper domain. These positively charged amino acids contact the negatively charged DNA strands and distort the DNA 1 to 4° (Avitahl and Calame, 1994).

α-Helices are stabilized by the amphipathic arrangement of their amino acid side chains that allow the formation of salt bridges (Landschulz *et al.*, 1988). The hydrophobic face of the α-helix facilitates a contiguous array of stabilizing interactions with another protein; this heptad repeat is a feature shared by proteins that dimerize in a coiled-coil

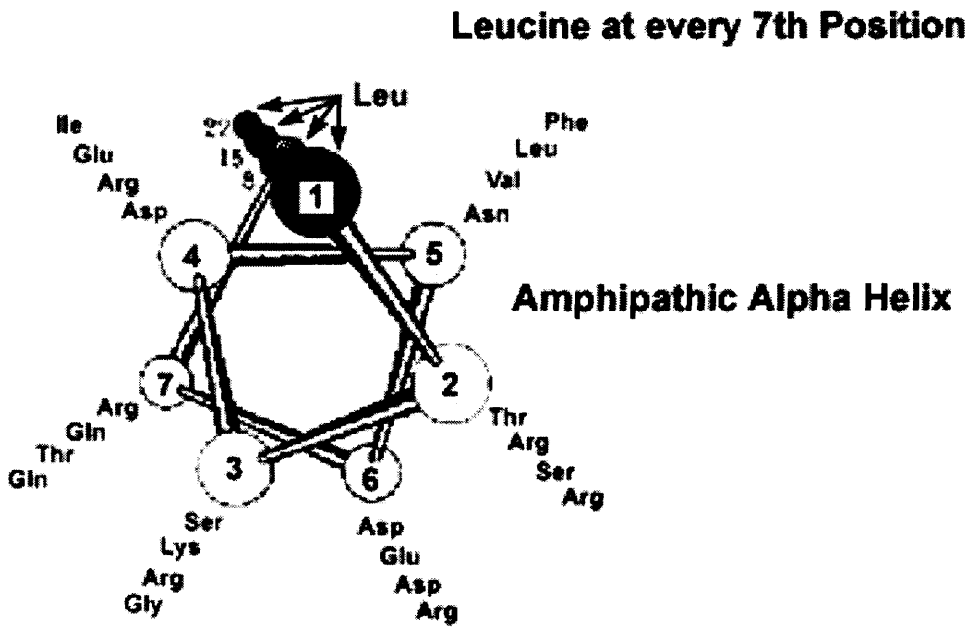


Figure 2.4: Helical wheel analysis of 28 amino acids of the C/EBP carboxy-terminus. Standard three letter amino acid nomenclature is utilized. Most notably, the Leu residues corresponding to residues 1, 8, 15 and 22 of the segments analyzed show a distinctive periodicity. This regular repeat of Leu residues or "leucine zipper" in conjunction with the hydrophobic amino acids at position 4, 12, *etc.* are proposed to act as a dimerization motif between separate polypeptides with similar leucine repeats, presumably via hydrophobic interactions.

motif (Krylov, 1994). The leucine zipper repeat allows proteins within the same family to interdigitate like a zipper thus stabilizing dimerization. Upon dimerization, the leucine zipper juxtaposes the basic region allowing it to contact DNA. Although the leucine zipper is not directly involved in contacting DNA, mutations within the leucine zipper motif inhibits dimerization making DNA binding impossible (Landschulz, *et al.*, 1988).

2.3.2.2 The Transactivation Domains of C/EBPα and C/EBPβ

The transactivation domains of inducible transcription factors are responsible for mediating constitutive and hormone inducible expression of a target gene. Using deletion analysis, several researchers observed that the N terminus of C/EBPα is composed of two distinct transactivation domains that are separated by a putative attenuator domain (Pei and Shih, 1991; Friedman and McKnight, 1990). In studying the effect C/EBPα has on the expression of the serum albumin gene in hepatoma cells, Nerlov and Ziff (1994; 1995) gained considerable insight into the constitutive activity of this transcription factor. Using N terminal and internal deletion mutants of C/EBPα's transactivation domain in transient transfection experiments, they identified regions of the protein that were

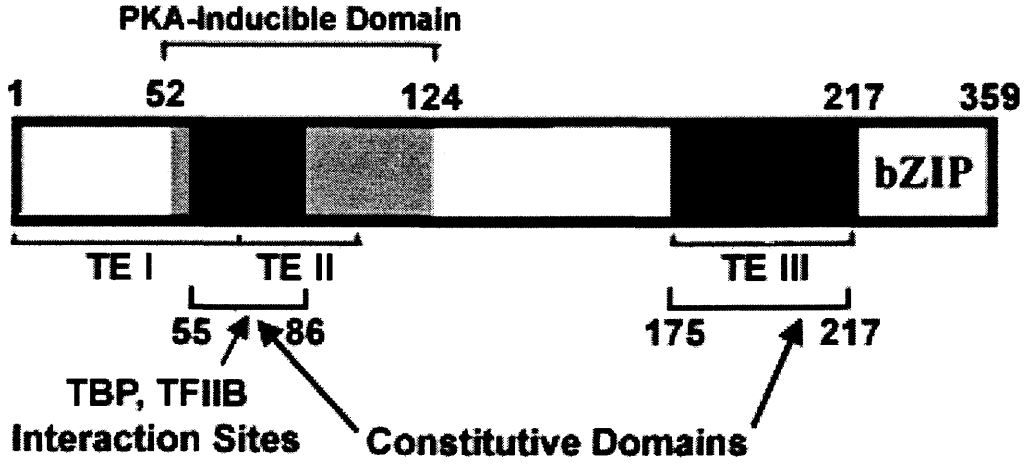


Figure 2.5: Schematic of the regions within C/EBPα protein responsible for mediating its constitutive and PKA-inducible response. The regions responsible for C/EBPα's constitutive and PKA-inducible activities have been assigned based on the studies of Nerlov and Ziff, 1994; 1995; Pei and Shih, 1991; Roesler *et al.*, 1998; and Wilson *et al.*, 2001. The numbers indicate the amino acid residues on the protein. The domains that mediate the constitutive activity of C/EBPα are shaded black, while the PKA-inducible domain is shaded gray. The region within C/EBPα that mediates protein-protein interactions with TATA binding protein (TBP) and TATA binding protein associated factor II B (TFIIB) are indicated. The basic region-leucine zipper DNA-binding motif resides at the carboxy-terminus of the protein and does not appear to contribute to its constitutive or PKA-inducible activity.

critical for mediating the constitutive activity of this protein. They indicated that C/EBPα has three transactivation elements that work in conjunction with each other to mediate the constitutive responsiveness of this protein. Deletion of any one of these elements did not significantly alter the basal responsiveness of C/EBPα, suggesting these elements are functionally redundant. However, deletion of two transactivation elements in any combination greatly diminished its basal activity. GST-pull down assays indicated that transactivation elements I and II were responsible for interactions with TBP and TFIIB, members of the general transcription apparatus (the regions of which are indicated by arrows in Figure 2.5), and deletion of these interaction sites abolished the constitutive activity of the protein. Transactivation element III has a strong negative regulatory domain and the amino acids responsible for the constitutive activity were spread throughout this transactivation element. This region binds chromatin remodeling complexes and it collaborates with the TBP and TFIIB interaction motifs during adipogenesis (Pedersen, *et al.*, 2001).

Roesler and colleagues performed deletion analysis on the C terminus of the transactivation domain of C/EBPα to identify the regions of the protein responsible for mediating its constitutive and PKA-

inducible responses. They identified that the PKA-responsive domain of C/EBPα resides within amino acids 50-124 (Roesler, *et al.*, 1998). Interestingly, mutations of amino acids 67, 77 and 78 in C/EBPα abolished the constitutive activity of the protein without affecting its ability to mediate the PKA response. This latter finding suggested that the constitutive and PKA-inducible activities of C/EBPα are mediated by different mechanisms and/or different domains. A major focus of this thesis is the study of the transactivation domain of C/EBPα and C/EBPβ to discern the domains within these proteins that are responsible for mediating their PKA-inducible response on the PEPCK promoter.

Trautwein and colleagues determined that the transactivation domain of C/EBPβ was composed of an acidic domain in the N terminus of the protein ranging from amino acids 21-105 and they suggested that amino acids 85-95 interact with the general transcription factors (Trautwein, *et al.*, 1995). Williams and colleagues delineated that the transactivation domain of mouse C/EBPβ contains two negative regulatory regions and three activation domain modules (Williams, *et al.*, 1991; 1995). These activation domain modules, like the transactivation elements of C/EBPα, have poor transactivation potential in isolation but they synergize to mediate a full transactivational response of C/EBPβ.

Studies by Roesler, *et al.*, (1998) indicated that PKA responsiveness of the PEPCK promoter was inhibited when GAL4-C/EBPβ was bound to the CRE site or when C/EBPβ was over-expressed in the presence of the endogenous PEPCK promoter. This GAL4-chimera consisted of the amino acids 8-315 of C/EBPβ (which includes a portion of the DNA binding domain) fused to the GAL4 DNA-binding domain. However, Park, *et al.*, (1999) showed that a truncated transactivation domain of C/EBPβ (amino acids 1-108) fused to a GAL4 DNA binding domain could mediate the PKA responsiveness of this protein on a permissive PEPCK promoter. The domain extending from amino acids 109-315 may contain a region that attenuates the PKA-inducible activity of C/EBPβ on the PEPCK promoter.

2.3.2.3 Multiple Translation Products of C/EBPα and C/EBPβ

Different isoforms of C/EBPα and C/EBPβ are generated via translation initiation at multiple in-frame AUG start codons. There are *five isoforms of rat C/EBPα expressed from five in-frame AUG start* codons which result in 42, 40, 30, 20 and 14 kD isoforms with the 42 and *30 kD isoforms being expressed to high levels in the liver (Greenbaum, et* *al.*, 1995; Hsieh, *et al.*, 1998). Like C/EBPα, C/EBPβ can be expressed as

multiple isoforms from its mRNA. Full length C/EBPβ (38 kD) and LAP (liver-enriched transcriptional activator protein) (35 kD) encodes the acidic activation domain as well as the highly conserved bZIP domain (Trautwein, *et al.*, 1995). A 21 kD isoform, LIP (liver enriched transcriptional inhibitory protein) and a 14 kD isoform both lack the region corresponding to the transactivation domain although they retain the ability to heterodimerize and contact DNA (Descombes and Schibler, 1991; Poli, *et al.*, 1990; Williams, *et al.*, 1995). Because the 21 kD and 14 kD isoforms lack the region corresponding to the transactivation domain, these proteins act as dominant negative proteins when they heterodimerize with other family members (Descombes and Schibler, 1991; Williams, *et al.*, 1995).

By initiating translation from several in-frame AUG start codons, different isoforms of C/EBPs are generated which have unequal portions of its transactivation domain. Using H4IIE cells and stable transfection analysis, Crosson and colleagues observed that while the overall mRNA level of C/EBPα was decreased in streptozotocin-induced diabetic rat liver, the 30 kD isoform was much more significantly reduced than the larger transcript (Crosson, *et al.*, 1997). These data suggest that in the diabetic rat liver, there is a shift towards the recognition of the first start

codon of C/EBPα over the second start codon. In other words, expression of a transcription factor's full-length or truncated transcripts can be potentially influenced in a hormonal or diseased state that affects the activity of the protein.

2.3.2.4 Regulation of C/EBPα and C/EBPβ DNA Binding by Phosphorylation

Because the bZIP domains of C/EBPα and C/EBPβ share such a significant degree of homology and they recognize the same *cis*-elements, there remains the question of how one isoform is selected to bind specific *cis*-elements on a gene promoter over another isoform. Obviously, increasing the expression level of an isoform and/or the more active variant of an isoform will allow this isoform a greater competitive advantage for binding to *cis*-elements. Studies have been undertaken to identify potential phosphorylation sites within the bZIP domain of each isoform to discern if the phosphorylation state of an isoform affects its ability to bind DNA. The bZIP domain of C/EBPα can be phosphorylated by Protein kinase C (PKC) on Ser^{248}, Ser^{277} and Ser^{299}, with the latter site significantly inhibiting its DNA binding ability *in vitro* but it is not clear if these Ser sites can be phosphorylated *in vivo* (Mahoney *et al.*, 1992).

However, it is known that the bZIP domain of C/EBPα is not a target for PKA (Mahoney, *et al.*, 1992).

C/EBPβ can be phosphorylated by PKA and PKC on Ser^{240}, a site within the basic region of the protein (Chinery, *et al.*, 1997). Phosphorylation of this site by either kinase abrogates its DNA-binding ability (Trautwein, *et al.*, 1995). These data offer an explanation of how the different isoforms may be selected for DNA binding. In the presence of PKA, the DNA binding domain of C/EBPβ may be phosphorylated, inhibiting its ability to interact with the *cis*-element and, by default, C/EBPα can bind to the *cis*-element without competition from C/EBPβ. Others, however, have suggested that C/EBPβ is not phosphorylated by PKA to any great extent *in vivo* (Wegner, *et al.*, 1992). Phosphorylation of C/EBPβ on Ser^{240} also stimulates the nuclear translocation of C/EBPβ from the cytosol which may aid in the protein's ability to outcompete other C/EBP isoforms for sites on the promoter (Chinery, *et al.*, 1997; Metz and Ziff, 1991). Obviously, the role of phosphorylation in affecting the *in vivo* DNA binding ability of C/EBPα and C/EBPβ must be much more extensively studied.

2.4 Mechanism of C/EBPα and C/EBPβ Transactivation Activity

C/EBPs have historically been considered as constitutively acting factors, unlike other transcription factors whose activities can be regulated by covalent modification, binding of a specific ligand, *etc.* in the presence of hormones. C/EBPα and C/EBPβ have several transactivation domains, some of which can physically interact with general transcription factors present in the preinitiation complex and some of which can mediate their PKA-inducible activities. Evidence which indicates that C/EBPs mediate the PKA-inducible response on the PEPCK promoter includes: (1) C/EBPs bind to several of the *cis*-elements that make up the CRU on the PEPCK promoter, (2) mutations in these sites which disrupt C/EBP binding also affect the fold-responsiveness to PKA, (3) over-expression of a dominant negative C/EBP inhibits PKA responsiveness of the PEPCK promoter in liver-derived cells, and (4) chimeric GAL4-C/EBPs, which have the DNA-binding domain of C/EBP replaced by the corresponding domain of the yeast transcription factor GAL4, can reconstitute the PKA responsiveness of PEPCK promoter variants which have one or more of the C/EBP sites substituted with binding sites for GAL4 (Liu, *et al.*, 1991; Park *et al.*, 1999; Roesler, *et al.*, 1994; 1996). By using GAL4-derived chimeras of C/EBP, it was ascertained that both C/EBPα and C/EBPβ can participate in the PKA-

inducible response and the domains within C/EBPs that participate in the constitutive and PKA-inducible activities could be analysed without interference from the endogenous C/EBP proteins within hepatoma cells. Moreover, since the GAL4 chimeras were constitutively expressed and localized to the nucleus, it appeared unlikely that the PKA-induced transcriptional activity of C/EBPs occurred via enhanced translocation to the nucleus from the cytosol or due to increased abundance of these proteins. Rather, the data are consistent with the hypothesis that C/EBPs contain a PKA-inducible domain, similar to that of CREB.

While it is well-established that C/EBPs can mediate PKA responsiveness by indirect mechanisms such as enhanced translocation of C/EBPs from the cytosol to the nucleus in response to elevated cAMP levels and enhanced expression of C/EBPs in response to cAMP, it is not generally appreciated that isoforms of C/EBPs possess domains which contain an intrinsic PKA-inducible activity independent of direct phosphorylation by PKA (Metz and Ziff, 1991; Pelletier, *et al.*, 1998). This intrinsic activity may only manifest itself within certain promoter contexts due to the involvement of other so-called "accessory" factors. Unlike CREB, C/EBPα is not a substrate for PKA and, to date, there have been no observations published that indicate C/EBPα interacts with any co-

activator molecules. There is evidence that CREB binding protein activates C/EBPα-dependent transcription and that these two proteins co-localize to the pericentromeric chromatin but there is no direct evidence that C/EBPα and CREB binding protein physically interact (Erickson, *et al.*, 2001; Schaufele, *et al.*, 2001). The N-terminus of C/EBPβ directly interacts with a homologue of CREB binding protein *in vivo* and cotransfection experiments indicate that this CREB binding protein homologue potentiates the activation of a C/EBP responsive gene by C/EBPβ (Mink, *et al.*, 1997). Exactly how C/EBP proteins induce or repress expression, how they interact with co-activators and how and if they are post-translationally modified to mediate their hormonal response has yet to be elucidated.

2.5 Objectives

The ultimate objective of this thesis is to identify the regions within the transactivation domains of C/EBPα and C/EBPβ that are required to mediate the PKA-inducible response and, to a lesser extent, the constitutive activity on the PEPCK promoter. A significant amount of information has been gathered about the regions of C/EBPα responsible for mediating its constitutive transcriptional activation of multiple gene

promoters. However, as the work in this thesis will outline, the regions of a transcription factor that are important for mediating the constitutive and hormone inducible response may be distinct. Many levels of regulation are required to control the complex milieu of metabolic processes in each tissue under multiple hormonal and dietary stimuli. This thesis will outline how different domains of C/EBPα mediate a PKA-inducible response depending on which *cis*-element it occupies on the PEPCK promoter. Not only do different transcription factors mediate hormonal responsiveness and constitutive activity via distinct domains, the domains of the transcription factor that mediate the inducible activity may vary depending on which *cis*-element it occupies on the promoter. This observation suggests another level of transcriptional regulation in the already extremely complex field of gene expression regulation.

The specific objectives of this thesis are:

(1) To define and characterize the amino acids within the C/EBPα and C/EBPβ transactivation domains that are required to mediate the PKA response on the PEPCK promoter within hepatoma cells.

(2) And, once the objective 1 is completed, to elucidate if the importance of the indicated amino acids are determined and/or influenced by the *cis*-element it occupies within the cAMP response unit on the PEPCK promoter.

3. MATERIALS AND METHODS

3.1 Reagents

All reagents used for this thesis were of molecular biology or analytical grade as appropriate. In Table 3.1, the names of the reagents and the suppliers are listed. Table 3.2 indicates the addresses of the individual suppliers from which the reagents in Table 3.1 were obtained.

Table 3.1: List of Reagents and Suppliers

Reagent	Supplier
Absolute Ethanol	BDH
Acetate	BDH
Acetyl-CoA	Pharmacia
Acrylamide	Bio-Rad
Agarose	Bio-Rad
Ammonium Persulphate	Bio-Rad
Ammonium Acetate	BDH
Aprotinin	Sigma
Adenosine Tri-Phosphate (ATP)	Boehringer Mannheim
Bio-RAD Protein Assay Dye Reagent Concentrate	Bio-Rad
Bovine Serum Albumin	ICN
Bromophenol Blue	Sigma

Reagent	Supplier
Calcium Chloride	BDH
Chloramphenicol	BDH
Chloroform	BDH
Coomassie Brilliant Blue Stain	Sigma
Diethylpyrocarbonate (DEPC)	BDH
Dinucleotide Triphosphate (dNTP)	Pharmacia
Dimethylsulfoxide (DMSO)	Sigma
2,5-Diphenyloxazole (PPO)	Sigma
Dithiothreitol (DTT)	Promega
Ethidium Bromide	Sigma
Ethylenebis (oxyethylenenitrilo) Tetraacetic Acid	BDH
Ethylene-Diamine Tetraacetic Acid Disodium Salt (EDTA)	BDH
Glacial Acetic Acid	BDH
D-Glucose	BDH
Glutathione-Sepharose Beads	Pharmacia
Glycerol	BDH
Glycine	BDH
Glycogen Azure	Sigma
Hydrochloric Acid (HCl)	BDH
N-2-Hydroethylpiperazine-N'-2 -Ethane Sulphonic Acid (HEPES)	USB
Isobutanol	BDH
Isopropyl-β-D-thiogalactopyranoside (IPTG)	USB
Isopropanol	BDH
Low Melting Point (LMP) Agarose	Bio-Rad
Magnesium Chloride ($MgCl_2$)	BDH
β-Mercaptoethanol	Sigma
Methanol	BDH
N,N'-Methylene—Bis-Acrylamide	Bio-Rad
Nonident NP-40	BDH
o-Nitrophenyl-β-D-Galactopyranoside (ONPG)	Boehringer Mannheim
Pepstatin A	Sigma
Phenol	Sigma
Phenylmethanesulfonyl Fluoride (PMSF)	Sigma

Reagent	Supplier
Phosphate Buffered Saline	BDH
1,4-Piperazine Diethane Sulfonic Acid, Sodium Salt (PIPES)	Sigma
Polyethylene Glycol (PEG)	Sigma
Potassium Chloride	BDH
Sodium Chloride (NaCl)	BDH
Sodium Bicarbonate (Na_2CO_3)	BDH
Sodium Deoxycholate	BDH
Sodium Dodecyl Sulphate (SDS)	Pharmacia
Sodium Hydroxide (NaOH)	BDH
Sodium Orthovanadate	Sigma
N,N,N',N'-Tetramethylenediamine (TEMED)	Sigma
Toluene	BDH
Tris-HCl	GIBO-BRL
Trypsin-EDTA	GIBCO-BRL
Tween-20	Sigma
Urea	BDH
Xylene Cylanol FF	Sigma
Zinc Chloride	BDH
Reagents for Bacterial Growth	
Ampicillin	ICN
Bacto-Agar	DIFCO
Bacto-Tryptone	DIFCO
Bacto-Yeast Extract	DIFCO
Kanamycin	Boehringer Mannheim
M13K07 Helper Phage	NEB
Cell Stock Freezing Media (DMSO)	GIBCO-BRL
Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (Ham)(1:1) –with Glutamine	GIBCO-BRL
Fetal Bovine Serum (Mexican)	GIBCO-BRL
Enzymes	
T4 DNA ligase	GIBCO-BRL
DNA Polymerase I, Klenow Fragment-Labeling Grade	Promega
RNAse A	Boehringer Mannheim

Reagent	Supplier
RNAse T_1	Boehringer Mannheim
RNasin	GIBCO-BRL
Taq DNA Polymerase- from Thermophilis Aquaticus	GIBCO-BRL
T7 RNA Polymerase	Promega
Restriction Endonucleases	NEB
Radioactive Substrates	
^{3}H-Acetyl-CoA	Perkin-Elmer
^{35}S-Methionine	Perkin-Elmer

Table 3.2: Names and Addresses of Reagent Suppliers

Supplier	Addresses
BDH	British Drug House, Saskatoon, Saskatchewan, Canada
Bio-Rad	Bio-Rad Laboratories, Mississauga, Ontario, Canada
Boehringer Mannheim	Boehringer Mannheim Canada Ltd., Laval, Quebec, Canada
DIFCO	DIFCO Laboratories, Detroit, Michigan, USA
GIBCO-BRL	Bethesda Research Laboratories, Burlington, Ontario, Canada
ICN	ICN Biomedical Canada Ltd., St. Laurent, Quebec, Canada
NEN-Mandel	Mandel Scientific Company, Guelph, Ontario, Canada
Perkin-Elmer	Perkin Elmer Life Sciences, Canada, Woodbridge ,Ontario, Canada
Pharmacia	Pharmacia LKB Biotechnology Ltd., Baie d'Urfe, Quebec, Canada
Promega	Fischer Scientific, Nepean, Ontario, Canada

Suppliers	Addresses
Santa Cruz Biotechnology	Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA
Sigma	Sigma Chemical Co., St. Louis, Missouri, USA
United States Biochemical (USB)	United States Biochemical Corporation, Cleveland, Ohio, USA

3.2 Bacterial Strains and Media Preparation

During the course of this thesis, *Escherichia coli (E.coli)* strain, NM522, was utilized for all cloning manipulations and propagation unless otherwise indicated. This strain was described in detail by Gough and Murray (1983) and Woodcock, *et al.*, (1989).

Luria-Bertani Ampicillin (LBA) plates were used to propagate the bacterial cells when they were transformed with an expression plasmid with ampicillin resistance. These plates were prepared by combining 10 g of bacto-tryptone, 5 g of bacto-yeast extract, 10 g of NaCl, and 15 g of agar in 1 L of double distilled water (dd$H_2$0.) This solution was sterilized via autoclavation for at least 20 minutes at 15 lb/sq. in. After the solution was sufficiently cooled, amphicillin was added to the medium to a final concentration of 50 μg/mL and the plates were poured.

LB liquid medium was used to prepare NM522 competent cells after overnight incubation at 37°C with nutation. This medium was prepared to the same final concentration of reagents as above up to a 200 mL volume except no antibiotic was added after sterilization and no agar was added to the medium.

Terrific Broth (TB) as described by Sambrook, *et al.*, (1989), was used to propagate bacterial cells in a liquid medium grown overnight at 37°C with nutation. This medium was prepared by combining 4.8 g of bacto-tryptone, 9.6 g of bacto-yeast extract and 1.6 mL of glycerol in 400 mL of ddH_20. This mixture was autoclaved as above. After the mixture cooled to a sufficient degree, a sterile solution of 0.17 M KH_2P0_4 and 0.72 M K_2HP0_4 (K-Salts) and the appropriate antibiotic were added to the medium using sterile technique.

K-Salts were prepared by combining 2.3 g of KH_2P0_4 and 12.54 g of K_2HP0_4 per 100 mL of ddH_20 and this mixture was autoclaved at 15 lb/sq. in. for 20 minutes.

3.3 General Molecular Cloning Procedures

All protocols in this section were at least partially based upon those presented in Sambrook, *et al.*, (1989). Unless otherwise specified, all centrifugation was performed at 12 500 rpm at room temperature.

3.3.1 Preparation of Competent Cells

An overnight culture was prepared from a single colony of *E. coli* inoculated into 5 mL of LB broth in a 50 mL sterile conical. The culture was grown overnight at 37°C with nutation to obtain a saturated bacterial solution. A 1:200 dilution of the saturated culture was used to inoculate 200 mL Luria Broth and a sample was removed to indicate the optical density (O.D.) of the culture at zero time. The culture was grown at 37°C with nutation until the culture reached an $O.D._{590}$ of 0.375. The culture was decanted into 4 equal volumes into 50 mL pre-chilled sterile conical tubes and incubated on ice for 10 minutes before being pelleted at 3000 rpm for 7 minutes at 4°C on a Clinical Centrifuge from the International Equipment Co. The supernatant was decanted and the cell pellets were resuspended in 10 mL of ice-cold $CaCl_2$ solution consisting of 15% glycerol, 60 mM $CaCl_2$ and 10 mM PIPES, pH 7.0. The washing step was repeated and the cell pellets were then suspended in 10 mL of $CaCl_2$

solution and incubated on ice for 10-30 minutes. The cell pellets were resuspended in 2 mL of $CaCl_2$ solution and dispensed into pre-chilled sterile tubes and stored at -80°C.

3.3.2 Transformation of Competent Bacterial Cells

An aliquot of 100 µL of competent cells was introduced to approximately 1 µg of plasmid DNA in a 12 mL falcon tube and incubated for 10-30 minutes on ice. The tubes were heat-shocked at 42°C for 2 minutes to allow for bacterial cell transformation. The heat-shocked cells were incubated on ice for 2 minutes before being plated on an LB agar plate with the appropriate antibiotic and incubated overnight at 37°C. Single colonies of bacteria were selected and propagated for small scale plasmid isolation and further diagnostics or genetic manipulation.

3.3.3 Small Scale Plasmid DNA Isolation

A 5 mL culture of Terrific Broth with the appropriate antibiotic was inoculated with a single *E. coli* colony and incubated overnight at 37°C with nutation. The protocol used to isolate small scale plasmid DNA was based upon the protocol of Lee and Rasheed, (1990). The plasmid DNA

pellet was resuspended in 25 μL 1X TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0) containing 0.1 μL of 10 mg/mL RNase.

3.3.4 Large Scale Plasmid DNA Isolation

A 400 mL culture of Terrific Broth with the appropriate antibiotic was inoculated with a 5 mL culture of a single *E. coli* colony (described above) and the 400 mL culture was incubated overnight at 37°C with nutation. The procedure used was based upon the alkaline lysis method described by Sambrook, *et al.*, (1989). The plasmid DNA pellet was resuspended in 0.1X TE to a concentration of 1 μg/μL as determined by acquiring the optical density at OD_{260} in which 1.0 corresponds to 50 μg plasmid DNA. A ratio of OD_{260}/OD_{280} which registered between 1.7 to 1.8, indicated a relatively pure plasmid preparation.

3.3.5 Agarose Gel Electrophoresis

Unless otherwise specified, agarose gel electrophoresis was typically preformed on a 1% agarose gel in 1 X TAE buffer and 0.5 μg/mL ethidium bromide. The running buffer was 1 X TAE consisting of 40 mM Tris-Acetate and 1 mM EDTA at pH 8.0. Samples were mixed with an appropriate volume of 5X agarose gel sample buffer consisting of 50%

glycerol, 50 mM EDTA, pH 8.0 and traces of bromophenol blue and xylene cylanol FF before being loaded onto the gel. Electrophoresis typically was carried out at between 90 and 110 volts until the appropriate band resolution was achieved.

3.3.6 Restriction Digests

Typically, digestion of DNA with restriction enzymes were carried out in 20 μL of ddH_2O and the reaction consisted of approximately 1 μg DNA, 2 μL of 10X buffers (appropriately selected according to the manufacturer's recommendations), and 1-2 U of each restriction endonuclease. The samples were incubated at 37°C (unless otherwise indicated by the manufacturer) for at least 1 hour and not more than 12 hours.

3.3.7 DNA Fragment Isolation and Ligation Reaction

After the appropriate band separation was achieved by agarose gel electrophoresis, a small section directly under the band of interest was *excised and the void was filled with 0.9% low melting-point agarose* (LMP) and 0.5 μg/mL ethidium bromide. After the LMP agarose congealed, the electrophoresis was reinitiated for the appropriate amount

Table 3.3: Plasmids Utilized For Gene Cloning

Plasmid Name	Cloning Vector	Cloning Site	Origin of Insert
C/EBPα	pBK-CMV	EcoRI-HindIII	18R-C/EBPα
C/EBPββ	pBK-CMV	EcoRI-Xba1	pM1-C/EBPββ
C/EBPαα	CMV-C/EBPαα	Pst1-Xba1	18R-C/EBPαα
C/EBPαβ	CMV-C/EBPαα	Kpn1-Xba1	CMV-C/EBPββ
C/EBPβα	pBK-CMV	EcoRI-Kpn1, Kpn1-Xba1	CMV-C/EBPαα, CMV-C/EBPββ
C/EBPβ	pBK-CMV	HindIII-Xba1	pBluescript II KS-NFIL6
GAL4-α60-217	pM2	BamHI-Pst1	19R-C/EBPα 6-60-217
GAL4-α72-217	PM2	BamHI-Pst1	19R-C/EBPα 6-72-217
GAL4-αΔCR1	GAL4-α50-217	BamHI-Pst1	pM2-C/EBPα 60-217
GAL4-αΔCR1,2	GAL4 α50-217	BamHI-Pst1	pM2-C/EBPα 72-217
GAL4-αΔCR3	GAL4-α50-217	BamHI-Pst1	GAL4-α 112-217
GAL4-αΔCR2	GAL4-α50-217	BamHI-Pst1	19R-C/EBPα 6-72-217
GAL4-βCR1	GAL4-β1-108	NaeI-SacII	Oligonucleotide
GAL4-βCR2	GAL4-β1-108	NaeI-SacII	Oligonucleotide
PM1-HA	pM1	BglII-EcoRI	pHA3

Plasmid Name	Cloning Vector	Cloning Site	Origin of Insert
PM2-HA	pM2	BglII-EcoRI	pHA3
PM3-HA	pM3	BglII-EcoRI	pHA3
HA-C/EBPα	pM1-HA	EcoRI-HindIII	CMV-C/EBPα
HA-C/EBPβα	pM1-HA	EcoRI-Xba1	CMV-C/EBPβα
HA-C/EBPαα	pM1-HA	EcoRI-Xba1	CMV-C/EBPαα
HA-C/EBPαβ	pM1-HA	EcoRI-Xba1	CMV-C/EBPαβ
HA-C/EBPββ	pM1-HA	EcoRI-Xba1	CMV-C/EBPββ
HA-α50-217	pM3-HA	EcoRI-Pst1 Pst-Xba1	GAL4-α50-217 GAL4-C/EBPα
HA-αΔCR1	pM1-HA	EcoRI-Sac1	GAL4-αΔCR1
HA-αΔCR1,2	pM1-HA	EcoRI-Sac1	GAL4-αΔCR1,2
HA-αΔCR2	pM1-HA	EcoRI-Sac1	GAL4-αΔCR2
HA-αΔCR3	pM1-HA	EcoRI-Sac1	GAL4-αΔCR3
HA-C/EBPβ	pM1-HA	HindIII-Xba1	GAL4-C/EBPβ
HA-βCR1	HA-C/EBPβ	EcoRI-Xba1	GAL4-βCR1
HA-βCR2	HA-C/EBPβ	EcoRI-Xba1	GAL4-βCR2
FGC-αTA	FG-CREB	EcoRI-Pst1	pM1-C/EBPα
FGC-α50-217	FG-CREB	BamHI-Pst1	GAL4-α50-217
FCG-αΔCR1	FG-CREB	EcoRI-Pst1	GAL4-αΔCR1
FCG-αΔCR2	FG-CREB	EcoRI-Pst1	GAL4-αΔCR2

Plasmid Name	Cloning Vector	Cloning Site	Origin of Insert
FCG-αΔCR3	FG-CREB	EcoRI-Pst1	GAL4-αΔCR3
FCG-αTM	FG-CREB	EcoRI-Pst1	GAL4-αTM
^{35}S -C/EBPα	pTZ-18R	BamHI-HindIII	RSV-C/EBPα
^{35}S -α50-217	pTZ-18R	BamHI-Pst1	FCG-αTA
^{35}S -αΔCR1	pTZ-18R	BamHI-Pst1	FCG-αΔCR1
^{35}S -αΔCR2	pTZ-18R	BamHI-Pst1	FCG-αΔCR2
^{35}S -αΔCR3	pTZ-18R	BamHI-Pst1	FCG-αΔCR3
^{35}S -αTM	pTZ-18R	BamHI-HindIII	FCG-αTM

The plasmids designed above, with the exception of pM1-HA, pM2-HA, pM3-HA, the FG-CREB constructs and the ^{35}S- constructs were designed as expression vectors for Western analysis, and transient transfection analysis. The pM1-HA, pM2-HA, pM3-HA and FG-CREB vectors were designed to act as parent vectors for the construction of the indicated plasmids in Table 3.3 and the ^{35}S chimeras were used for *in vitro* protein expression. All plasmids were subjected to diagnostic restriction digests and/or automated DNA sequencing to verify the identity of each construct. The pM1, pM2 and pM3 vectors were described in Sadowski and Ptashne, (1989).

The parent plasmid used to construct CMV-C/EBPα was 18R-C/EBPα that was mutated via Stratagene's QuikChange Mutagenesis technique to introduce a Kpn1 restriction endonuclease site at the region of the gene corresponding to amino acid 255 via the primers 5'GGCTTGGCTGGTACCCACCCCGAACCTC3' and 5'GAGGTCGGGGT-GGGTACCAGCCAAGCC3' (ID Labs.) The Kunkel method of site-directed mutagenesis was used to introduce a Kpn1 restriction endonuclease site into 19R-NFIL6 at the region of the gene corresponding to amino acid 252 via primer 5'GCCTGCTACGCGGGTACCGGGC-CGGCGCCCTCGCAG3' (UCDNA.) The region of the genes corresponding to C/EBPα's and NFIL6's transactivation domain (TA) and DNA-binding domains (DBD) were now able to be isolated from each other. The plasmids with these Kpn1 sites between the TA and DBD were now identified as 18R-C/EBPαα and 19R-C/EBPββ, respectively. The 18R-C/EBPα plasmid insert was ligated in-frame to the pBK-CMV vector (Stratagene) via EcoRI-HindIII ligation to create C/EBPα. C/EBPα was subsequently restricted with Pst1-Xba1 and ligated with a Pst1-Xba1 digest of 18R-C/EBPαα insert to create C/EBPαα.

To engineer the C/EBPαβ vector and C/EBPββ, the 19R-C/EBPββ vector was restricted with HindIII-BamHI, introduced into pM1. This

vector was subsequently rerestricted and the insert was religated into the pBK-CMV vector via EcoRI-Xba1 digestion to create C/EBPββ. To isolate the β DBD, the C/EBPββ vector was restricted with Kpn1-Xba1 and this insert was ligated in-frame to C/EBPαα to create the plasmid, C/EBPαβ.

To create the plasmid C/EBPβα, a double ligation was performed. The C/EBPαα vector was restricted with EcoRI-Kpn1 to isolate the C/EBPα TA in soft agar and the C/EBPββ vector was restricted with Kpn1-HindIII and the C/EBPβ DBD was isolated in soft agar. The CMV vector was restricted with EcoRI-HindIII and isolated in soft-agar as well. The ligation reaction was typical (Section 3.3.7) except the 7 μL of insert consisted of 3.5 μL of C/EBP α TA (EcoRI-Kpn1) and 3.5 μL of C/EBP β DBD (Kpn1-Xba1) to construct the plasmid, C/EBPβα.

To create C/EBPβ, 19R-NFIL6 was restricted with HindIII-BamHI and ligated into pBluescript KS II (United States Biochemical). This vector was subsequently digested with HindIII-Xba1 and ligated into the CMV vector to create CMV-C/EBPβ.

In the next series of C/EBPα deletion constructs, the TA domains of C/EBPα and C/EBPβ were compared using the computer program INTERALIGNv1.0, Stanford and three regions with significant homology were identified. Plasmids were engineered to remove each conserved

region (CR) within C/EBPα's TA alone and in conjunction with the first 50 amino acids of C/EBPα. pM1-C/EBPα 6-217 was restricted with EcoRI-Pst1 and introduced into the pTZ-19R vector (United States Biochemical). This phagemid was used as a template to introduce BamHI sites via the Kunkel method into the sites corresponding to amino acids 60 and 72 using the primers 5'GACCCGGCCGGATCCAACGAC3' and 5'GAGCACGAGGGATCCATAGAC3', respectively (UCDNA). The mutant 19R-C/EBPα 6-217 constructs were digested with BamHI-Pst1 and ligated in-frame to pM2 to create GAL4-α60-217 and GAL4-α72-217. Because pM1, pM2 and pM3 have the region corresponding to the GAL4 DBD upstream of its multiple cloning site, the proteins expressed using these plasmids have the GAL4 DBD at its N terminus.

To construct the C/EBPα internal deletion mutants, a GAL4-C/EBPα construct which contains a BamHI site on the 3' end of the region corresponding to the first 50 amino acids (Roesler, *et al.*, 1998).was used as the ligation vector, (GAL4-α50-217). GAL4-α60-217 and GAL4-α72-217 were digested with BamHI-Pst1 and the inserts were ligated to the BamHI-Pst1 sites of the GAL4-α50-217 vector to create GAL4-αΔCR1 and GAL4-αΔCR1,2. A pM1-C/EBP construct consisting of the amino acids 112-217 from C/EBPα (Roesler, *et al.*, 1998) was digested with BamHI-Pst1

and the insert was ligated to GAL4-α50-217 to create the GAL4-αΔCR3 expression construct. Finally, the 19R-C/EBPα mutant with the BamHI restriction endonuclease site at the region corresponding to amino acid 60 was restricted with EcoRI-BamHI and the insert was ligated to the pM1 vector. This construct was subsequently restricted with BamHI-Pst1 and ligated with the insert corresponding to the amino acids 72-217 to create the plasmid GAL4-αΔCR2.

The C/EBPβ mutant GAL4-C/EBPβ was described in Park, *et al.* 1999. GAL4-βCR1 and GAL4-βCR2 were created by restricting the parent construct with NaeI-SacII and replacing the insert with oligonucleotide primers with extensive mutations at the regions corresponding to CR1 or CR2. The ID Lab primers which created GAL4-βCR1 were 5'GGCGGCGGGCGCGAGCGGCTCCAGGTAGGGGCTGAAGTCGATG GCGCGCGCGGCCGCGCCGGCGGCC3' and 5'GGCCGCCGGCGCG-GCCGCGCGCGCCATCGACTTCAGCCCCTACCTGGAGCCGCTCGCG CCCGCCGCCGC3'. The primers used to create GAL4-βCR2 were 5' GGCGGCGGGCGCGAGCGGCGCCGCGGCGGGGGCGGCGGCGACGG CGCGCTCGTGCTCGCCGATGGCC3' and 5'GGCCATCGGCGAGC-ACGACGCCGCCGTCGCCGCCGCCCCCGCCGCGGCGCCGCTCGCGC CCGCCGCCGC3'.

In the next series of mutant constructs, the majority of the above constructs were ligated in-frame to a plasmid constructed to contain a region which corresponds to the two adjacent hemagglutinin (HA) tags. The pM1, pM2 and pM3 plasmids were used as templates and their GAL4 coding regions were excised via a BglII-EcoRI digest and the vector was isolated in soft-agar. Using the pHA_3 vector (designed by Charest, *et al.* 1995), two PCR primers were created to hybridize with the region upstream of the Kozak sequence of HA_3 and introduce a BglII site at the 5' end of the forward primer (5'GACCGGAAGATCTCA-CCATGATCTTTTAC3') and the second primer was designed to introduce an EcoRI site between the second and third HA site of pHA_3 (5'ATATGGATAGAATTCTGCATAGTCCGGGACGTC3' (ID Labs.) After PCR amplification of this region, the PCR product was electrophoresed on a 1% agarose gel and isolated from soft-agar with an Agarose Gel DNA Extraction Kit (Boehringer Mannheim) as per the manufacturer's instructions. The purified HA_2 PCR product was restricted with BglII-EcoRI and the digest was ligated in-frame to each pM vector fragment creating the vectors, pM1-HA, pM2-HA and pM3-HA.

To create HA-C/EBPα and HA-C/EBPβα, the CMV-C/EBPα vector and CMV-C/EBPβα vectors were digested with EcoRI-HindIII and ligated

into the pM1-HA vector. HA-C/EBPαα, HA-C/EBPαβ, HA-C/EBPββ and HA-C/EBPβ were created by restricting the corresponding CMV parent vectors described above with EcoRI-Xba1 and ligating the inserts into pM1-HA.

The C/EBPα deletion constructs GAL4-αΔCR1, GAL4-αΔCR1,2, GAL4-αΔCR2, GAL4- ΔCR3 were digested with EcoRI-Sac1 and isolated in soft agar. Each insert was ligated to the HA-C/EBPα vector. HA-α50-217 was created by ligating the EcoRI-Pst1 TA fragment of GAL4-α50-217 to the pM3-HA2 vector. This vector was subsequently restricted with Pst1-Xba1 and ligated with the Pst1-Xba1 C/EBPα DBD insert. To create HA-C/-EBPβ, HA-βCR1 and HA-βCR2, the corresponding GAL4-C/EBPβ mutants were restricted with EcoRI-Xba1 and ligated into pM1-HA.

In order to create the *in vitro* expressed proteins, a series of FGC constructs had to be made. GAL4-αΔCR1, -αΔCR2, -αΔCR3, C/EBPα and C/EBPα TM were restricted with EcoR1-Pst1 and each insert was ligated into the FG-CREB vector (Roesler laboratory, unpublished data). FGC-α50-217 was created by restricting GAL4-α50-217 and introducing this insert into FG-CREB. To create ^{35}S -C/EBPα, ^{35}S -αΔCR1, ^{35}S -αΔCR2, ^{35}S -αΔCR3, and ^{35}S -α50-217, the respective FGC-C/EBPα constructs were restricted with BamHI-PstI and these DNA fragments are introduced into

the pTZ-18R vector. To create ^{35}S -C/EBPαTM, the FGC-αTM vector was restricted with BamHI-HindIII and this DNA fragment was introduced into pTZ-18R.

3.5 Culture of Human Hepatoma HepG2 Cells

Human hepatoma HepG2 (ATCC HB8065) cells were propagated at 37°C and 5% CO_2 in Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (Ham) (1:1)- with L-glutamine and containing 10% Fetal Bovine Serum. The cell line was propagated twice a week via trypsinization at a 1:4 or 1:5 dilution on 100 mm tissue culture plates.

HepG2 stocks of 100 mm tissue culture plates were prepared by trypsinizing the plates and resuspending the detached hepatoma cells in 10 mL of D-MEM cell media. The cells were centrifuged at 3000 rpm at 4°C for 5 minutes, the supernatant was removed and replaced with 1 mL of cell stock freezing media containing fetal bovine and calf serum, and 10% DMSO. The cells were subsequently frozen down in Liquid Nitrogen.

3.6 Transient Transfection of Human HepG2 Cells

Transient transfection of HepG2 cells was carried out as indicated by Sambrook *et al.*, (1989). Four hours before transfection, the adherent cells on a 100 mm plate were trypsinized and split into eight 60 mm plates or four 100 mm plates to render them approximately 70% confluent. The cells were transfected with the appropriate expression vectors, RSV-βgal, pTZ-19R and pMT-C where appropriate, to a total amount of 12.5 μg/plate. The transfection mixture was composed of 95 μL of 2 M $CaCl_2$ and 375 μL of 2X HBS (1.1915 g HEPES, 1.636 g NaCl, 0.2162 g Glucose, 0.5 mL of 2 M KCl, and 1.5 mL of 0.1 M $Na_2HPO_4.2\ H_20$ in 100 mL H_20) and 625 μL of ddH_20. The solutions and DNA were allowed to incubate for 20 minutes to form a fine $CaCl_2$-DNA precipitate before 500 μL was dispensed to each plate. After the cells were incubated at 37°C with 5% CO_2 overnight, the medium was aspirated and the plates were rinsed with PBS. D-MEM media plus 80 μM ZnCl was added to the plates and they were allowed to incubate at 37°C and 5% CO_2 for 24 hours.

3.6.1 Transient Transfection Analysis

The medium was aspirated and the adherent cells were rinsed with 1X PBS before 0.5 mL of Harvesting solution (40 mM Tris-HCl, pH 7.5, 1

mM EDTA, 150 mM NaCl) was added and the cells were scraped with a rubber policeman and kept on ice. The cells were transferred to a 1.5 mL eppendorf tube and quickly centrifuged to pellet the cells. The Harvesting solution was aspirated and the cells were resuspended in 150 μL 0.25 M Tris-HCl, pH 7.8. The cells were vortexed briefly and subjected to three freeze-thaw cycles at –80°C/37°C, respectively. The cells were centrifuged at 12 500 rpm at 4°C for 10 minutes and a 15 μL aliquot and a 3.5 μL aliquot was removed for β-galactosidase assay and protein analysis. The remainder of the cell suspension was heat-treated at 65°C for 5 minutes to inactivate competing enzymes for the Chloramphenicol Acetyltransferase (CAT) assay and aliquots of 30 μL and 6.5 μL were removed for the CAT assay and CAT protein analysis.

3.6.2 β-galactosidase Assay

A solution consisting of 66 μL o-Nitrophenol-β-D-galactopyranoside (ONPG) (4 mg/mL ONPG in 0.1 M Sodium Phosphate buffer, pH 7.5), 3 μL of 100X $MgCl_2$ (0.1 M $MgCl_2$ and 5 M β-mercaptoethanol) and 201 μL of 0.1 M Sodium Phosphate buffer, pH 7.5 (41/9 ratio of 0.1 M Na_2HPO_4 and 0.1 M NaH_2PO_4, respectively) was added to each 15 μL sample. The samples were incubated in a 37°C water bath

until a yellow, soluble product was formed. This process was timed from the point of water bath incubation of the samples until 500 µL of 1 M Na_2C0_3 was added to the yellow product. The absorbance of each sample was measured at 420 nm and the data was recorded.

3.6.3 CAT Assay

The CAT mix was composed of 10 µL of 5 mM chloramphenicol (dissolved in 0.25 mM Tris-HCl, pH 7.5), 1.5 µL of ^{3}H-Acetyl-CoA, 0.075 µL of 20 mM Acetyl-CoA, and 8.425 µL of 75 mM HCl. This 20 µL volume was added to 30 µL heat-treated samples and the samples were incubated for up to 3 hours in a 37°C water bath. The reaction was stopped upon the addition of 0.5 mL of 7 M Urea.

The samples were introduced to 10 mL of Toluene containing 0.8% 2,5-Diphenyloxazole (PPO) and the samples were incubated for 15 minutes at room temperature and subsequently counted on a Beckmann LS 6500 Scintillation Counter for 1.0 minute.

3.6.4 Protein Quantitation of Transfected Cells

A 1 mL volume of Bio-RAD Protein Assay Dye Reagent Concentrate diluted 1/4 with ddH_20 was added to each protein sample

and the samples were incubated at room temperature for 5 minutes before being read on a spectrophotometer at $O.D._{595}$. A concentration standard curve of Bovine Serum Albumin (BSA) ranging from 0-10 μg/mL Bio-RAD Reagent was subjected to spectrophotometric analysis to identify the slope of the standard curve.

3.7 Determination of Protein Expression via Western Analysis

3.7.1 Preparation of HepG2 Cell Nuclear Extracts

The transfected tissue culture HepG2 cells (Section 3.5) were rinsed with 1X PBS, harvested with a rubber policeman and pelleted. The cell pellet was resuspended in 400 μL cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) and the cells were allowed to swell on ice for 15 minutes. Subsequently, 25 μL of 10% Nonidet NP-40 was added, the tube was vortexed and centrifuged for 30 seconds at 12 500 rpm. The nuclear pellet was resuspended in 50 μL of ice cold buffer C (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1mM DTT and 1 mM PMSF). The tube was vigorously rocked at 4°C for 30 minutes, the nuclear extract was pelleted and the supernatant was introduced to a fresh tube. Aliquots of the supernatant fraction were subjected to SDS-PAGE analysis.

3.7.2 Transient Transfection Analysis Using Lipofectamine Reagent

Four hours before transfection, the adherent cells on a 100 mm plate were trypsinized and split into four 100 mm plates to render them approximately 70% confluent. A solution of 300 μL of D-MEM medium was incubated with 5 μg of lipofectamine reagent (Invitrogen) and 6 μg of the expression plasmid. The solutions and DNA were allowed to incubate for 20 minutes before being dispensed to each plate. After the cells were incubated at 37°C with 5% CO_2 overnight, the medium was aspirated and the plates were rinsed with PBS. D-MEM medium was added to the plates and they were allowed to incubate at 37°C and 5% CO_2 for 24 hours.

3.7.3 Recovery of Protein Lysates After Lipofectamine Transient Transfection

After transfection of tissue culture cells with the appropriate expression plasmid and RSV- β galactosidase, the confluent, adherent cells were rinsed with cold 1X PBS (140 mM NaCl, 27 mM KCl, 100 mM Na_2HPO_4-7 H_20 and 18 mM KH_2PO_4) before being scraped from the plates with 200 μL RIPA buffer (1 X PBS, 1% Nonidet-NP-40, 0.5% (w/v) sodium deoxycholate, 0.1 % SDS, 100 μg/mL PMSF, 2 μg/mL aprotinin, 1 mM sodium orthovanadate and 1 μg/mL pepstatin A.) Lysates were

centrifuged at 12 000 rpm for 15 minutes at 4°C, the supernatant was transferred to a fresh tube. An appropriate volume of 5X SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 400 mM 2-mercaptoethanol, 10% glycerol and traces of bromophenol blue) was added to the sample The concentration of protein was quantified by β-galactosidase assay.

3.7.4 Electrophoresis of Protein Lysates for SDS-PAGE and Western Blotting

Protein lysates were resolved on a 12% SDS-PAGE gel composed of 12% acrylamide: N,N'-methylene-bis-acrylamide (29.2:0.8), 375 mM Tris-HCl, pH 8.8, and 0.1% SDS in the resolving gel and 5 % acrylamide: N, N'-methylene-bis-acrylamide (29.2:0.8), 130 mM Tris-HCl, pH 6.8, and 0.1 % SDS in the stacking gel. The gel was polymerized following the addition of 0.1% APS and 0.04% TEMED. The running buffer was composed of 25 mM Tris-HCl, 250 mM glycine and 0.1% SDS. After the protein lysates had been properly quantified, approximately 20 μg was loaded onto the SDS-PAGE gel on a Bio-Rad Mini Protean® II Apparatus at and run at 100 volts until the bromophenol blue dye had runoff the gel.

The separate bands were then transferred onto a nitrocellulose or PVDF membrane using a Bio-Rad Trans-blot apparatus at 120 volts for 1

hour at 4°C. The transfer buffer was composed of 39 mM glycine, 48 mM Tris-HCl, and 20% methanol. The membranes were then rinsed with *blocking buffer (1X PBS and 0.04% Tween-20 (PBST)) and 5% Bio-Rad* Blotting Grade Blocker Non-Fat Dry Milk for 1 hour at room temperature. The blocked membrane was then incubated with 1:1000 of the appropriate 1° antibody (Santa Cruz, Biotechnology) in blocking buffer overnight at 4°C with gentle nutation. After overnight hybridization, the membrane was washed extensively with blocking buffer and subsequently incubated with a 1:10 000 dilution of the appropriate 2° antibody conjugated to horseradish peroxidase (Santa Cruz, Biotechnology) for 1 hour at room temperature. The membrane was again extensively washed in blocking buffer and finally it was washed twice with PBST. The antibody-protein complexes were detected using the NEN-Mandel Renaissance® chemiluminescent detection kit and Kodak X-OMAT film at room temperature.

3.8 Site-Directed Mutagenesis

3.8.1 Stratagene's QuikChange™ Site-Directed Mutagenesis

Oligonucleotide primers were designed to perfectly hybridize with the same sites on the sense and antisense template except for a mutation

introduced at the center of the primers. A PCR reaction was designed as per the manufacturer's instructions. Upon completion of the thermocycling, 1 μL of the restriction enzyme Dpn 1 was introduced to each sample for 1 hour at 37°C to cleave the methylated parent strands specifically. At least 1 μL of the Dpn1 treated DNA samples were transformed in NM522 cells and plated onto LB plates with the appropriate antibiotic. Colonies were selected and tested for the mutations via restriction analysis or DNA sequencing.

3.8.2 Kunkel Method of Site-Directed Mutagenesis

3.8.2.1 Preparation of Single-Stranded DNA

In order to perform the Kunkel method of site-directed mutagenesis, the parent phagemid was transformed into CJ236$^{dut\text{-}ung\text{-}}$ *E. coli* to create uracil-containing DNA. A colony was selected and grown in 5 mL of 2XYT media (16 g bacto-tryptone, 10 g bacto-yeast, and 5 g NaCl autoclaved as above) and 50 μg/mL ampicillin at 37°C in a shaking incubator. A 50 mL 2XYT + ampicillin culture was inoculated with 500 μL of the saturated culture and 200 μL of M13K07 phage and the culture was incubated at 37°C with shaking for 1 hour. After this time, a final concentration of 75 μg/mL Kanamycin was added to the overnight culture.

To isolate the single stranded, uracil containing nucleic acid (ssDNA), the culture was pelleted at 7 000 g on a Clinical Centrifuge from the International Equipment Co. for 15 minutes at 4°C. The supernatant was transferred to a fresh tube and 1/4th volume of 3.5 M NH_4-Acetate and 20% PEG solution was introduced and the solution was incubated on ice for 30 minutes. The centrifugation step was repeated, the pellet was resuspended in 200 µL of 1 XTE and the sample was extracted twice with phenol and once with chloroform to remove impurities. The single stranded DNA was precipitated with 1/10th volume of 3 M NaOAc (pH 5.2) and 2 volumes of absolute ethanol. The single stranded DNA was rinsed with 70% ethanol and allowed to air-dry before being resuspended in 1X TE to a concentration of 1 µg/mL.

3.8.2.2 Performing Kunkel Method Site-Directed Mutagenesis

To perform the mutagenesis reaction, 1 µg of ssDNA, 10 pmol of phosphorylated oligonucleotide containing the mutation, 1 µL of 10X PE1 buffer (200 mM Tris, pH 7.5, 100 mM $MgCl_2$, 500mM NaCl, 10 mM DTT) up to 10 µL dd$H_2$0 were combined and heated for 5 minutes at 20°C above the Tm of the primer. The sample was allowed to cool slowly to allow proper primer-template hybridization. Next, 10 µL of PE3 buffer (1 µL of

PE2 buffer (200 mM Tris, pH 8.0, 100 mM $MgCl_2$, 10 mM DTT), 1 μL of 10 mM dNTP's, 1 μL of 10 mM ATP, 1 μL of 5 mM ligase, 2 U of Sequenase up to 10 μL H_20) was added to the sample and it was incubated for 2 hours at room temperature before being transformed into NM522 cells and plated on LBA plates overnight at 37°C. Colonies were selected and tested for the mutation by restriction analysis or sequencing.

3.9 Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) mixture consisted of 10 pmol of both the forward and reverse primers, 2 μL of 50 mM $MgCl_2$, 2 μL of 10 mg/mL dNTP's, 5 μL of 1X PCR buffer, 1 Unit of Taq DNA polymerase, and approximately 100 mmol template in 50 μL ddH_20. The thermocycling program typically consisted of an initial denaturation step at 94°C for 5 minutes, followed by another denaturation step at 94°C for 30 seconds, an annealing step at 55°C for 59 seconds, an extension step at 72°C for 30 seconds and the program wraps to the second denaturation step for 30 more cycles after which the samples are incubated at 4°C. The PCR products were visualized via ethidium bromide staining on a 1-2% agarose gel.

3.10 *In Vitro* Protein-Protein Interaction

3.10.1 GST-Fusion Protein Induction

A 400 mL TBA culture was inoculated with a 1:10 dilution of overnight saturated cultures of BL21 *E.coli* (Pharmacia) transformed with either pGEX-2T or the pGEX-2T recombinant fusion proteins. The cultures were incubated for 1 hour at 37°C with nutation. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 1 mM and growth continued at 37°C with nutation for 4 hours.

For fusion protein recovery, bacterial cell cultures were pelleted by centrifugation at 5000 * g for 10 min at 4°C and a portion of the pellet was resuspended in 1 mL RIPA buffer. The bacteria were lysed on ice by sonication and centrifuged at 10 000 * g for 5 min at 4°C. The aliquots were incubated with 200 uL glutathione-Sepharose beads that had been previously washed three times with NETN buffer (20 mM Tris, pH8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40) containing 0.5% powdered milk. The bacterial supernatant and glutathione-Sepharose beads were incubated for 30 min at 4°C with rocking. The glutathione-Sepharose beads and fusion proteins were washed three times with NETN buffer.

3.10.2 Production of ^{35}S-Labeled Protein Using Promega's TNT *In Vitro* Transcription/Translation Kit™

Plasmid DNA was prepared as described in Section 3.5.3. A solution of 25 μL of Rabbit Reticulocyte Lysate was incubated with 1 μg plasmid DNA, 1 μL of amino acid mixture minus methionine, 2 μL of ^{35}S-Methionine, 0.2 μL RNasin, 1 μL T7 RNA Polymerase, 2 μL TNT buffer, up to 50 μL with DEPC treated H_20. The mixture was incubated for 90 min at 30°C.

3.10.3 GST-Pull Down Assay

A volume of 10 μL of the radiolabeled protein was incubated for 1 hour at 4°C with 100 μL of NETN washed-glutathione-Sepharose beads and the volume was brought up to 200 μL NETN. The glutathione-Sepharose beads were washed three times with NETN buffer and the beads were resuspended in 75 μL SDS-PAGE sample buffer and boiled for 5 minutes. The samples were centrifuged for 5 minutes at 4°C and a volume of 12 μL was loaded onto a 12% SDS-PAGE gel. The gel was dried at 80°C for 2 hours under vacuum and the gel was exposed to autoradiography.

4. RESULTS

4.1 "Domain Swapping" Analysis of C/EBPα and C/EBPβ

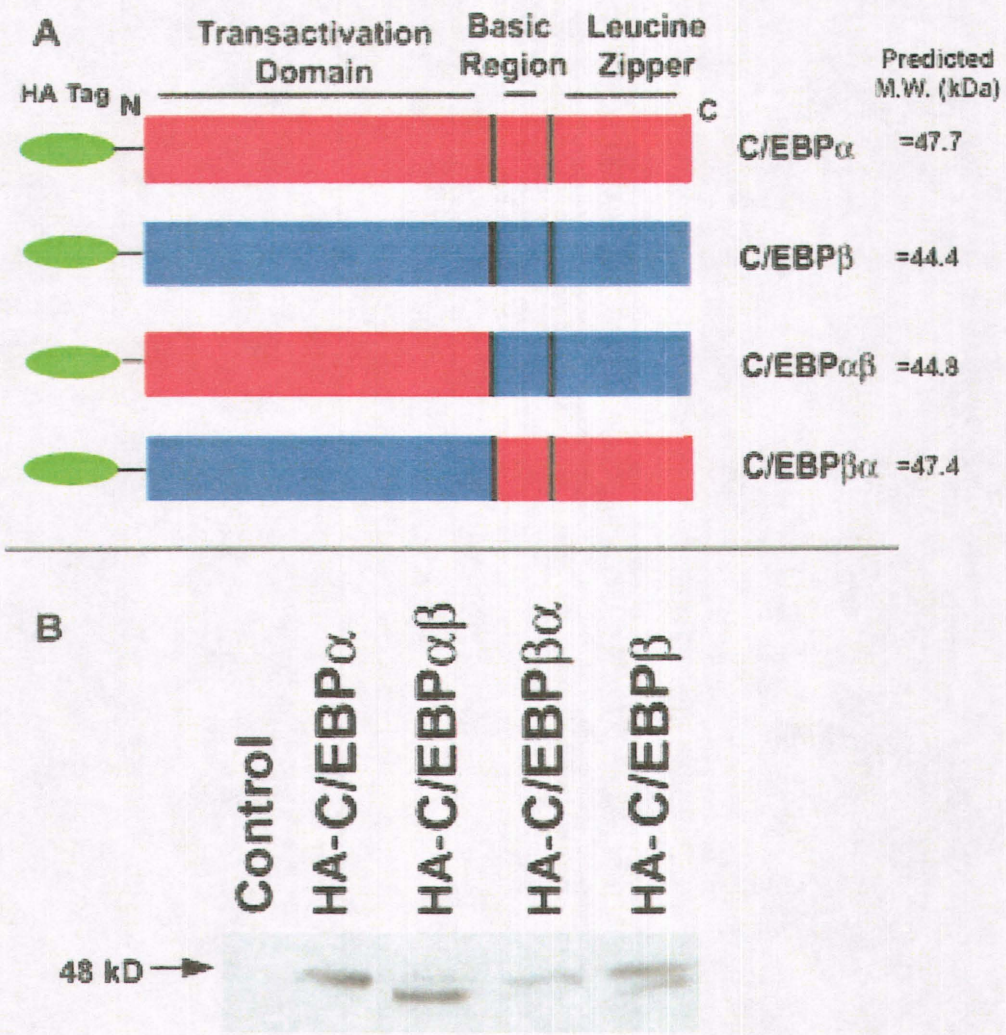
4.1.1 Rationale for bZIP "Domain Swapping" Analysis of C/EBPα and C/EBPβ

Previous co-transfection analysis of the endogenous PEPCK promoter and a plasmid coding for the C/EBPα or C/EBPβ protein indicated that, unlike C/EBPα, C/EBPβ was not capable of mediating a PKA-inducible response in HepG2 cells (Roesler, *et al.* 1998). However, when a significant portion of the C terminal region of C/EBPβ was deleted (as described in Park, *et al.* 1999), GAL4-C/EBPβ$_{1\text{-}108}$ was able to mediate the PKA-inducible response when it was tethered to the distal sites on the PEPCK promoter. This finding suggested that the amino acids 109-315 may contain a region that attenuates the ability of C/EBPβ to mediate a PKA-inducible response on the PEPCK promoter. Alternatively, the bZIP domain of each isoform, though highly conserved, may exert unique regulatory properties onto their respective transactivation domains.

In order to evaluate the effect that the bZIP domain of C/EBPα and C/EBPβ may exert on their respective transactivation domains, the bZIP domains of C/EBPα and C/EBPβ were "swapped". Site-directed mutagenesis was used to incorporate a Kpn1 site at the site corresponding to amino acid 255 and amino acid 252 of C/EBPα and C/EBPβ, respectively. The chimera C/EBPαβ is composed of the transactivation domain of C/EBPα linked to the DNA binding domain of C/EBPβ, while the C/EBPβα chimera is composed of the transactivation domain of C/EBPβ linked to the DNA binding domain of C/EBPα (Figure 4.1A). The proteins were expressed with a HA tag fused to its N terminus so that the expression level of each protein could be evaluated by Western analysis without interference from endogenous C/EBP proteins in HepG2 cells.

Western analysis was performed to verify that the proteins were expressed at comparable levels in HepG2 cells (Figure 4.1B). The control lane represents HepG2 cells that were transiently transfected with pM1-HA (a vector that expresses the HA-tag). Due to the small size of the expressed HA-tag, it was run off the gel and therefore does not appear on the blot. As indicated in the control lane, the HA antibody did not interact with any non-specific proteins at approximately 48 kD. Although the

Figure 4.1: "Domain swapping" analysis of C/EBPα and C/EBPβ. A) Schematic of the HA-tagged C/EBP bZIP domain "swap" mutants. To create the "swap" mutants, the DNA binding domain of C/EBPα and C/EBPβ proteins were "swapped" or exchanged. C/EBPαβ is composed of the transactivation domain of C/EBPα fused to the DNA binding domain of C/EBPβ whereas C/EBPβα is composed of the transactivation domain of C/EBPβ fused to the DNA binding domain of C/EBPα. B) Western analysis of HA-C/EBP "swap" mutants. Transient transfection analysis was performed as indicated in Section 3.7.2. After 48 hrs, the cells were processed as per Section 3.7.3. Equal concentration of proteins were loaded and electrophoresed on a 12% SDS-PAGE gel and transferred onto a nitrocellulose membrane. The control lane represents HepG2 cells that were transiently transfected with pM1-HA (a vector that expresses the HA tag). The "swap" mutants were detected with an antibody against the HA tag. The 48 kD molecular weight marker is indicated.


A
Transactivation Domain
Basic Region
Leucine Zipper
HA Tag
N
C
Predicted M.W. (kDa)
C/EBPα =47.7
C/EBPβ =44.4
C/EBPαβ =44.8
C/EBPβα =47.4
B
Control
HA-C/EBPα
HA-C/EBPαβ
HA-C/EBPβα
HA-C/EBPβ
48 kD

molecular weight of the transactivation domains of C/EBPα and C/EBPβ are almost identical, the bZIP domain of C/EBPα is slightly longer than the bZIP domain of C/EBPβ. Therefore, C/EBPαβ has a slightly smaller molecular weight than the C/EBPα wild type protein and C/EBPβα has a slightly larger molecular weight than the C/EBPβ wild type protein. Although a band is present at the approximate predicted molecular weight in lane C/EBPβ, there is also a slightly faster migrating band. The identity of this faster migrating band is unknown but DNA sequencing and restriction analysis of the HA-C/EBPβ plasmid (and the other HA-"swap" mutants) verified the integrity of the plasmid. It may be that the faster migrating band represents a C/EBPβ protein that has been phosphorylated by PKA on Ser 105 and/or Ser 299. The HA-tagged C/EBP proteins were expressed to comparable levels in HepG2 cells and the indicated proteins migrate to the appropriate predicted molecular weight.

4.1.2 Effect of C/EBP bZIP "Domain Swapping" on its Constitutive and PKA-Inducible Activity.

HepG2 cells were transiently transfected with expression plasmids for the C/EBPα and C/EBPβ "domain swap" mutants to study the effect that these domains have on the PKA response in the presence of the CAT

reporter gene, -490PCK (as depicted in Figure 2.2). In this and every transient transfection analysis performed in this thesis, an expression plasmid which codes for the catalytic subunit of PKA is co-expressed into the cell line to mimic the effect in a cell whereupon cAMP has triggered the release of the catalytic subunit of PKA from the regulatory domain. C/EBPα and its respective "swap" mutant (C/EBPαβ) as well as C/EBPβ and its respective chimera (C/EBPβα) showed comparable constitutive activity, which suggests that the bZIP domain does not affect the constitutive activity of the transactivation domain. Over-expression of C/EBPα enhanced the PKA responsiveness of the PEPCK promoter, as did over-expression of either of the two swapped mutants, C/EBPαβ and C/EBPβα (Figure 4.2). However, over-expression of C/EBPβ showed little, if any, ability to enhance the PKA responsiveness of the PEPCK promoter over that seen in HepG2 cells where no C/EBPβ protein was over-expressed ('None' lane). Clearly the transactivation domain of C/EBPβ can mediate a strong PKA-inducible response (as shown by C/EBPβα) but the bZIP domain of C/EBPβ appears to exert a negative effect on its transactivation domain. In summary, the respective bZIP domains did not affect either chimera's ability to mediate constitutive activity but the

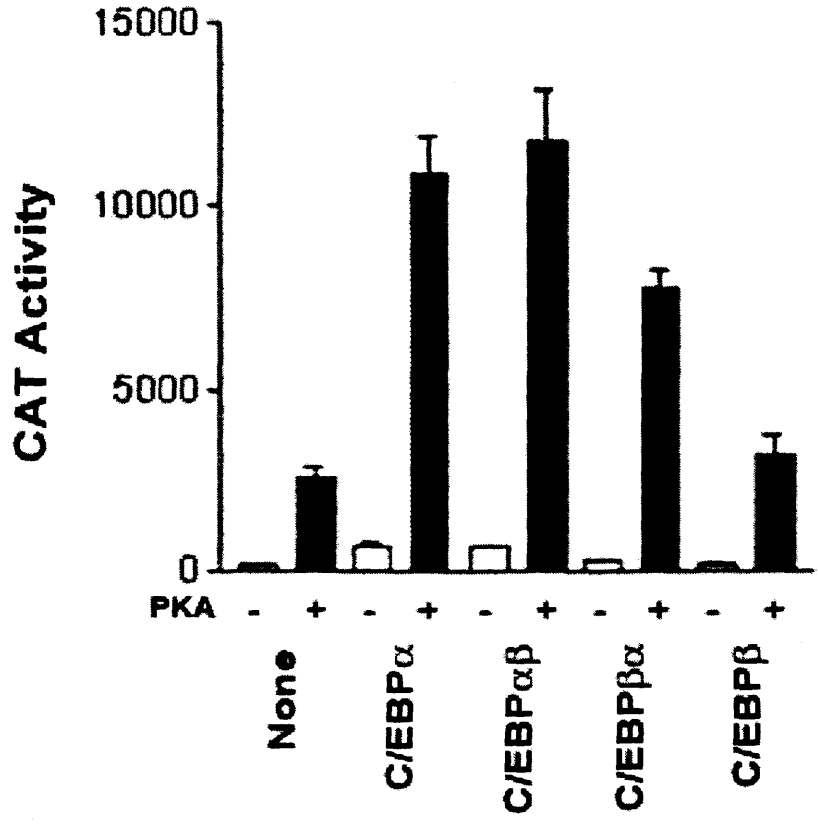


Figure 4.2: Effect of bZIP "domain swapping" between C/EBPα and C/EBPβ. HepG2 cells were transiently transfected with 3.5 μg of –490PCK in the absence (clear bars) and presence (solid bars) of a PKA expression vector (1 μg), and 1 μg of each C/EBP mutant, as indicated. The control lane represents HepG2 cells that were not transiently transfected with C/EBP expression plasmids. The values shown are the means +/- S.E. of at least three experiments.

bZIP domain of C/EBPβ did negatively affect the ability of C/EBPβ's transactivation domain to mediate a PKA-inducible response.

4.1.3 Rationale for Leucine Zipper "Domain Swapping" Analysis of C/EBPα and C/EBPβ

Williams and colleagues "swapped" the C/EBPα leucine zipper domain for the C/EBPβ leucine zipper domain and vice versa to create the chimeric proteins C/EBPαβLZ and C/EBPβαLZ (Figure 4.3) (Lee, *et al.* 1997b). They determined that expression of a liver specific P-450 gene was up-regulated when C/EBPβ, but not C/EBPα, occupied sites on its proximal promoter in HepG2 cells. "Domain swap" experiments revealed that the leucine zipper and the transactivation domain of C/EBPβ were absolutely required to induce transcription of the P-450 gene. These chimeric C/EBP proteins were obtained from the Williams' lab and they were tested for their ability to induce transcription on the endogenous PEPCK promoter in HepG2 cells in the presence and absence of PKA.

4.1.4 Effect of Leucine Zipper "Domain Swapping" on the Activity of C/EBPα and C/EBPβ.

All of the C/EBP proteins tested in this experiment were shown to induce PEPCK promoter activity in the absence of PKA by approximately 2 fold (Figure 4.4). These data were consistent with the constitutive activity of the wild type C/EBPα and C/EBPβ proteins on the -490PCK promoter (Roesler, *et al.* 1998). C/EBPα and its chimera, C/EBPαβLZ, showed comparable PKA-inducible activity suggesting that the leucine zipper domain does not affect the PKA-inducible activity of the protein. Similarly, the C/EBPβ protein and its chimera (C/EBPβαLZ) also showed strong PKA-inducible activity. The leucine zipper domains of C/EBPα and C/EBPβ did not alter the ability of either C/EBPα or C/EBPβ's transactivation domain to mediate a constitutive or PKA-inducible activity of the PEPCK promoter in HepG2 cells.

One should note that, in Figure 4.4, the C/EBPβ protein mediated a strong response to PKA on the PEPCK promoter whereas, in Figure 4.2, C/EBPβ failed to mediate a PKA-inducible response. Although the reason for this discrepancy is not immediately obvious, the answer may lie in the

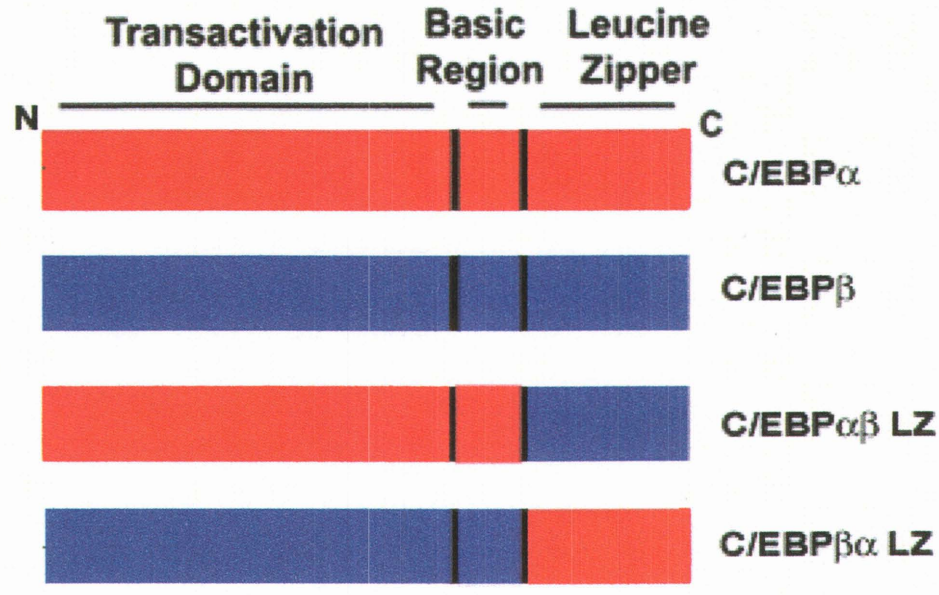


Figure 4.3: Schematic of the C/EBP leucine zipper "swap" mutants. The DNA binding domain of C/EBPα and C/EBPβ proteins have been "swapped" or exchanged. C/EBPαβLZ is composed of the transactivation domain and the basic region of C/EBPα fused to the leucine zipper of C/EBPβ whereas C/EBPβαLZ is composed of the transactivation domain and basic region of C/EBPβ fused to the leucine zipper domain of C/EBPα.

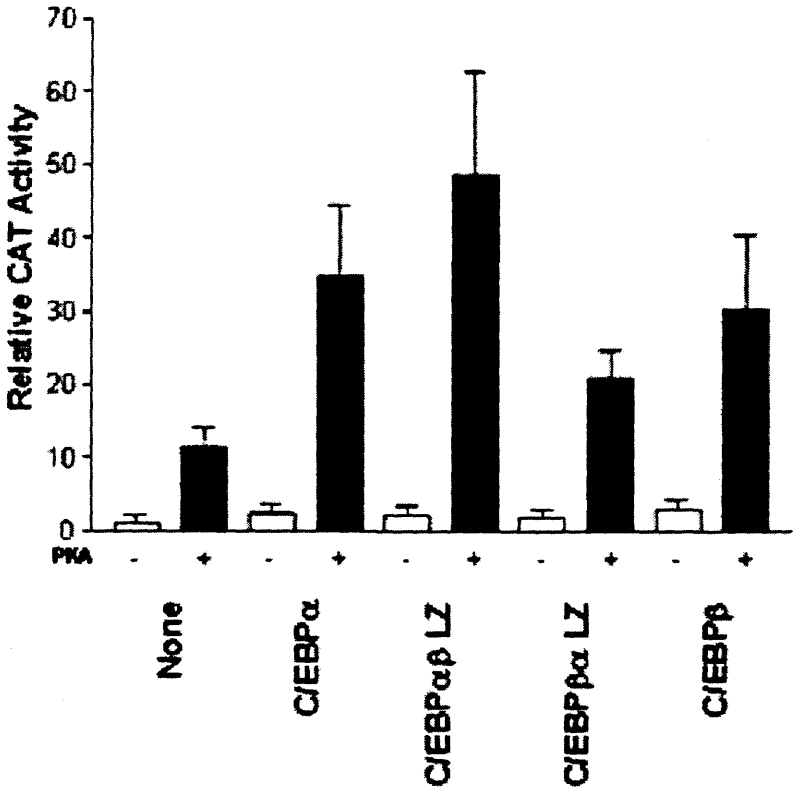


Figure 4.4: Effect of leucine zipper "domain swapping" on C/EBP α and C/EBPβ activity. HepG2 cells were transfected with 7 μg of -490 PCK, 500 ng of each indicated plasmid and 1 μg of PKA expression plasmid. The relative CAT activity was calculated as the ratio of the CAT activity measured in the presence and absence of PKA expression vector co-transfection and these experiments are the +/- S.E. of at least three experiments. The control lane represents HepG2 cells that were not transiently transfected with C/EBP expression plasmids.

fact that two different expression vectors were used to generate these proteins. The expression vector used to generate protein in Figure 4.2 was driven by an RSV promoter and the C/EBPβ gene was derived from the human species. The vector used to generate protein in Figure 4.4 was driven by a CMV promoter and the gene was derived from the mouse model. Use of different expression vectors (with different promoters) and genes from different animal models (with corresponding differences in the 5′ untranslated region and different possible translation start site) may alter the overall expression level or activity of a protein.

4.2 Structure/Function Analysis of C/EBPα

4.2.1 Rationale for Site-Specific Deletion Analysis of C/EBPα

Structure/function analysis of C/EBPα by a number of different researchers have identified regions within the transactivation domain that mediate constitutive and PKA-inducible activity (Figure 2.3) (Nerlov and Ziff, 1994; 1995; Friedman and McKnight, 1990; Pei and Shih, 1991; Roesler, *et al.* 1998). Studies by Park, *et al.* (1999) indicated that residues 1-108 of C/EBP were required to mediate the PKA-inducible activity on the PEPCK promoter. Comparison between the PKA-responsive transactivation domains of these two family members identified three

conserved regions (CRs) (Figure 4.5). In the experiments described below, the contribution of these CRs to the PKA-inducible activity of C/EBPα was explored.

Fusion proteins composed of the GAL4 DNA binding domain fused to C/EBPα transactivation domain or the indicated C/EBPα deletion mutants were constructed (Figure 4.6). C/EBPα N-terminal deletion mutants were created which sequentially deleted the first 50 amino acids, CR1, CR2, and the amino acids between CR2 and CR3. C/EBPα internal deletions mutants were also constructed as indicated. Because the transactivation domain of C/EBP proteins are functionally independent from the DNA binding domain, fusion of the GAL4 zinc finger to the N terminus instead of the C terminus of the protein has no deleterious effect on the activity of the transactivation domain. Therefore, expression of a GAL4-fusion protein can determine the potential a protein has to mediate a constitutive or hormone inducible response on a permissive promoter without interference from the endogenous proteins expressed in the cell line.

The GAL4 fusion protein expression levels were verified by Western analysis using a GAL4-specific antibody (Figure 4.7). Control lanes indicate HepG2 cells that were transiently transfected with the

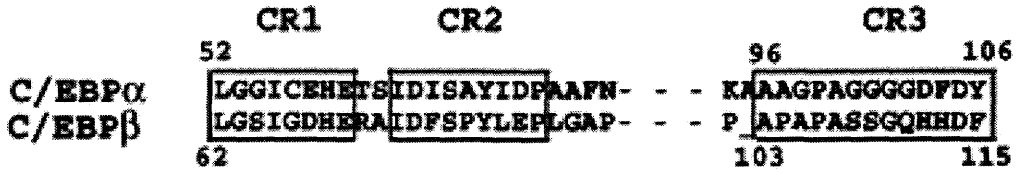


Figure 4.5: Amino acid homology between C/EBPα and C/EBPβ. A computer based multiple alignment analysis was performed on the regions of C/EBPα and C/EBPβ previously shown to be necessary to mediate the PKA-inducible response on the PEPCK promoter. Three regions of homology (boxed areas) were identified and termed CR1, CR2 and CR3. The corresponding amino acid residues are numbered.

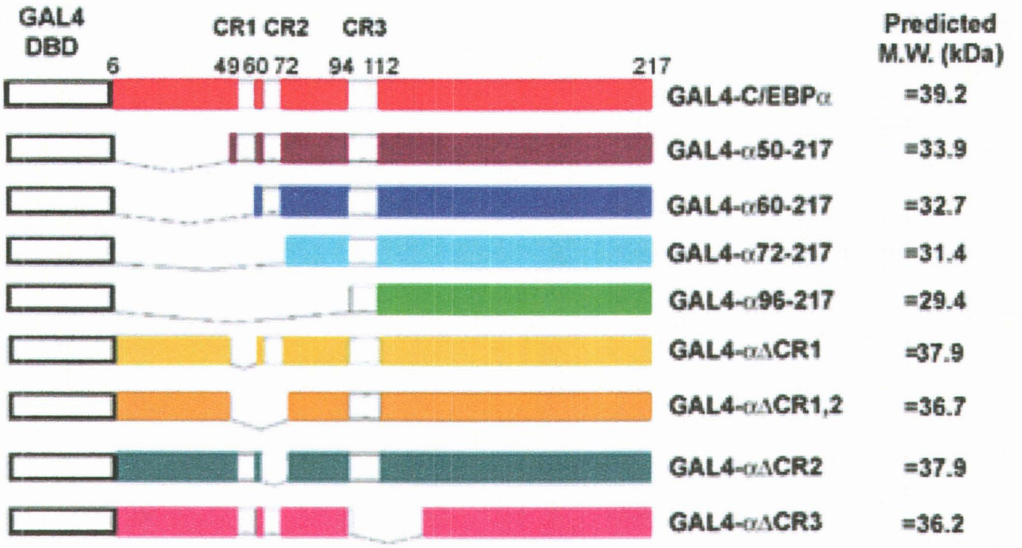


Figure 4.6: Schematic of the GAL4-C/EBPα fusion proteins. GAL4-C/EBPα contains the entire rat C/EBPα N terminal transactivation domain (minus the first 5 amino acids) linked to the DNA binding domain of the yeast transcription factor GAL4. The remaining constructs have modifications to the transactivation domain as shown but are otherwise similar to GAL4-C/EBPα. Numbers above GAL4-C/EBPα indicate the amino acid positions on the C/EBPα transactivation domain. The predicted molecular weight (M.W.) of each protein is indicated.

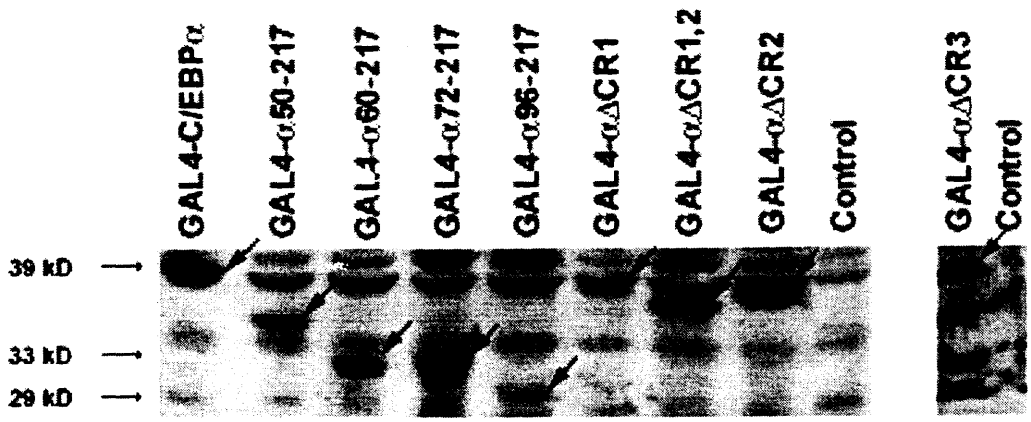


Figure 4.7: Western analysis of GAL4-C/EBPα deletion mutants. Transient transfection analysis was performed in HepG2 cells as indicated in Section 3.6. After 48 hrs, a nuclear extract assay was prepared and 70 μg of each extracts was resolved on a 12% SDS-PAGE gel and transferred onto a nitrocellulose membrane. The proteins were detected with an antibody against the GAL4 DNA binding domain. Arrows indicate the over-expressed GAL4-fusion proteins and molecular weight markers are shown.

plasmid pM1 which expressed the GAL4 domain. In order to achieve sufficient separation of the protein bands, the GAL4-domain was run off the gel and thus did not show up in the controls lanes. Several non-specific bands were identified by comparison with the control lanes. Identity of over-expressed proteins over non-specific bands was achieved by examining the intensity of the bands compared to control lanes. Although all the proteins were over-expressed, the intensity of each band did vary slightly. For instance, the intensity of the GAL4-αΔCR1 band is only approximately 2 times brighter than the non-specific band of approximately similar molecular weight in the adjacent GAL4-α96-217 lane. Although GAL4-αΔCR3 and GAL4α72-217 proteins did migrate to the appropriate approximate position on the gel, these two proteins did migrate to a slightly higher position than predicted. However, the remaining GAL4-proteins migrated to the appropriate position on the gel relative to the molecular weight standards. Multiple restriction enzyme diagnostics and sequencing were also performed on each plasmid construct to verify that each gene was the correct size and nucleotide sequence (data not shown).

4.2.2 Effect of N Terminal Deletion Mutants on C/EBPα When it is Tethered to Distal Sites on the PEPCK promoter

Initially, the constitutive activity of the C/EBPα N-terminal deletion constructs were evaluated in the presence of the artificial PEPCK promoter -109/G3A1, which consists of the five *cis*-elements that constitute the CRU except that the three C/EBP binding sites were replaced with GAL4 binding sites (Figure 4.8; Roesler *et al.* 1996). Deletion of the first 50 amino acids of C/EBPα's transactivation domain (GAL4-α50-217) did not affect the constitutive activity (Figure 4.9), although further deletion to residue 60, which resulted in the removal of CR1 (GAL4-α60-217), reduced the protein ability to mediate constitutive activity by approximately 55% relative to GAL4-C/EBPα. Deletion of the N-terminal transactivation domain up to residues 72 or 96 (GAL4-α72-217, GAL4-α96-217) of C/EBPα had no additional effect on the constitutive activity of the protein (Figure 4.9).

The ability of the GAL4 fusion protein to mediate a PKA-inducible response was evaluated using the same promoter. Although deletion of amino acids 6-49 did not reduce the constitutive activity of C/EBP, GAL4-α50-217 showed a reduced, yet still robust, response to PKA compared to GAL4-C/EBPα. When the residues in GAL4-C/EBPα's transactivation

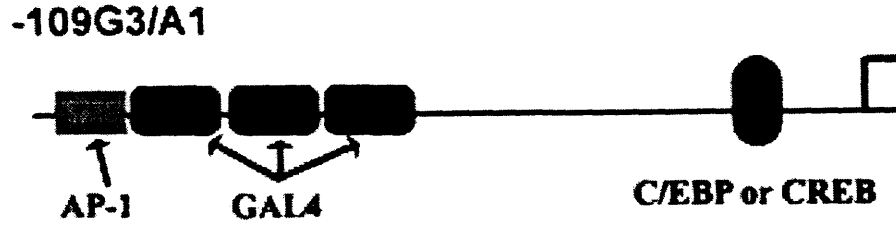


Figure 4.8: Schematic of the artificial PEPCK promoter, -109/G3A1. This artificial PEPCK promoter reconstitutes the CRU with the exception that GAL4 sites replace the three C/EBP binding sites and the relative position of AP-1 has been altered.

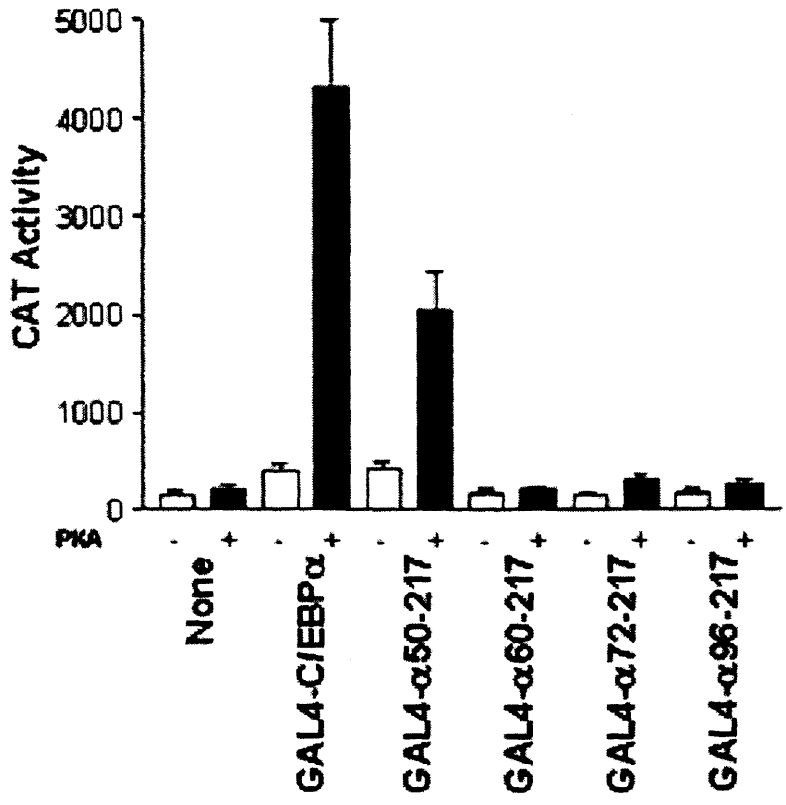


Figure 4.9: Effect of over-expression of C/EBPα N terminal deletion mutants in the presence of –109/G3A1. HepG2 cells were transiently transfected with 3.5 μg of -109/G3A1 in the absence (clear bars) and presence (solid bars) of a PKA expression vector (1 μg) and 1 μg of each GAL4-C/EBPα N terminal deletion mutant, as indicated. The control lane represents HepG2 cells that were not over-expressed with a GAL4-C/EBPα expression plasmid. The values shown are the means +/- S.E. of at least three experiments.

domain up to and including CR1 (GAL-α60-217) or both CR1 and CR2 were removed (GAL4-α72-217), an almost complete loss of constitutive and PKA-inducible activity was observed (Figure 4.9).

4.2.3 Effect of Internal Deletion Mutations on C/EBP Activity When Tethered to Distal Sites on the PEPCK Promoter

The next step in the analysis was to specifically remove the conserved regions within the context of the intact transactivation domain of C/EBPα in order to assess the effect these amino acids have on the constitutive and PKA-inducible activities on the PEPCK promoter. Internal deletion mutants GAL4-αΔCR1, GAL4-αΔCR1,2, GAL4-αΔCR2 and GAL4-αΔCR3 (shown in Figure 4.6) were tested by transient transfection analysis in HepG2 cells in the presence of the reporter plasmid, -109/G3A1. Deletion of CR1 or CR3 did not appear to affect the constitutive activity of the fusion protein although deletion of CR1 and CR3 did reduce the PKA-inducible activity relative to GAL4-C/EBPα, the mutants retained substantive, albeit lower, PKA-inducible activity (Figure 4.10). Mutants lacking CR2 (GAL4-αΔCR1,2 and GAL4-αΔCR2) lost all constitutive activity as well as most of the PKA-inducible activity. (Figure 4.10).

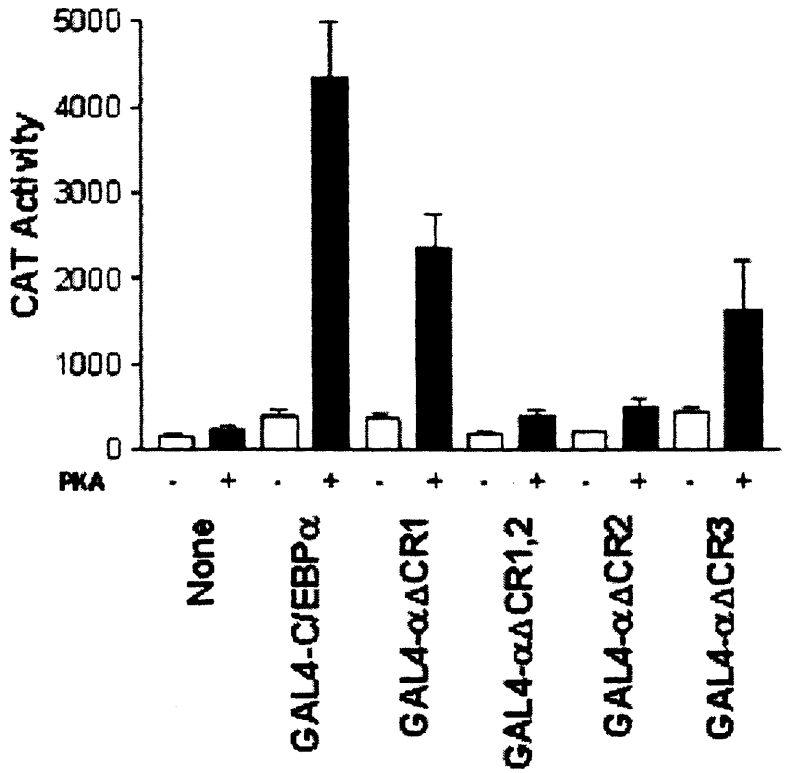


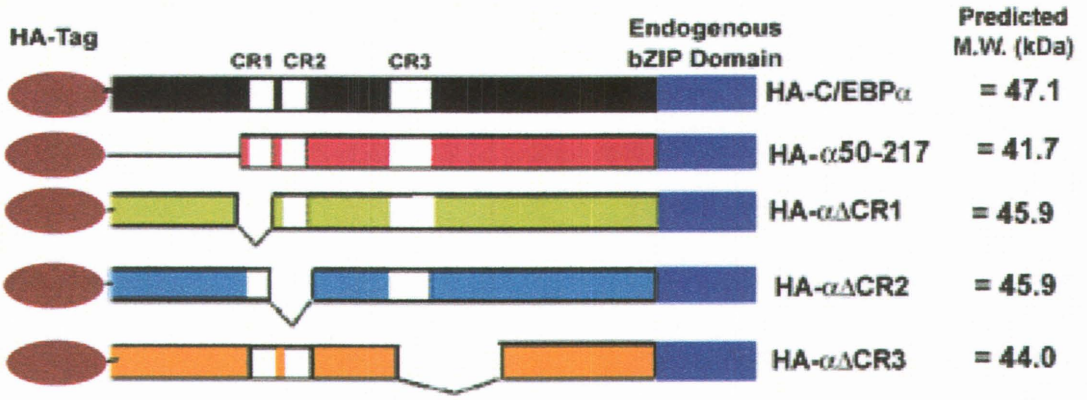
Figure 4.10: Effect of over-expression of internal GAL4-C/EBPα deletion mutations in –109/G3A1. HepG2 cells were transiently transfected with 3.5 μg of -109/G3A1 in the absence (clear bars) and presence (solid bars) of a PKA expression vector (1 μg) and 1 μg of each GAL4-C/EBPα internal deletion construct, as indicated. The control lanes represent cells that *have not been transfected with a GAL4-C/EBPα expression plasmid. The* values shown are the means +/- S.E. of at least three experiments.

4.2.4 Assessment of the Ability of C/EBP Mutants to Act as Dominant Negative Mutants

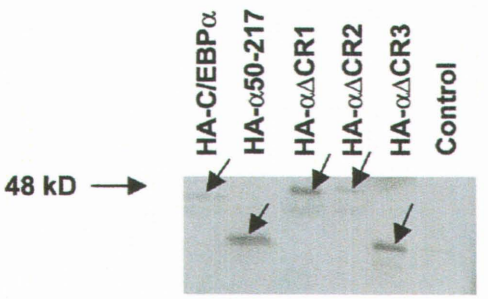
The data in Sections 4.2.2 and 4.2.3 indicate that CR2 is a critical domain within C/EBPα for mediating the constitutive and PKA-inducible activity on the artificial PEPCK promoter. It was hypothesized that over-expression of full-length C/EBPα with a deletion in CR2 may be able to inhibit the PKA responsiveness of the PEPCK promoter due to a dominant-negative type of mechanism. HepG2 cells are an appropriate cell line in which to test this hypothesis since these cells have relatively low level of C/EBPα expression compared to primary hepatocytes which should enhance the dominant negative action of the over-expressed mutants (Friedman *et al.*, 1989). Figure 4.11A depicts the full-length C/EBPα and the corresponding deletion mutants ligated in-frame to a HA tag (Charest, *et al.*, 1995).

Figure 4.11: HA-C/EBPα Deletion Mutants A) Schematic of HA-C/EBPα deletion proteins. HA-C/EBPα contains the entire rat C/EBPα N terminal transactivation domain (minus the first 5 amino acids) linked to its endogenous DNA binding domain and it is covalently attached to two HA tags at its N terminus. The remaining mutants depicted have modifications to the transactivation domain as shown but are otherwise similar to HA-C/EBPα. Predicted molecular weight (M.W.) of each protein is indicated. B) Western analysis of HA-C/EBPα mutants. Transient transfection analysis was performed on HepG2 cells as indicated in Section 3.7.2. After 48 hrs, the cells were processed as indicated in Section 3.6. Equal concentrations of protein were loaded and electrophoresed on a 12% SDS-PAGE gel and transferred onto a nitrocellulose membrane. The deletion mutants were detected with an antibody against the HA tag. The molecular weight markers are indicated.

A


HA-Tag
CR1 CR2
CR3
Endogenous
bZIP Domain
Predicted
M.W. (kDa)
HA-C/EBPα
= 47.1
HA-α50-217
= 41.7
HA-αΔCR1
= 45.9
HA-αΔCR2
= 45.9
HA-αΔCR3
= 44.0

B


HA-C/EBPα
HA-α50-217
HA-αΔCR1
HA-αΔCR2
HA-αΔCR3
Control
48 kD

Western analysis, using an HA-specific antibody, was performed to verify that the proteins were expressed to comparable levels in HepG2 cells (Figure 4.11B). The control lane represents HepG2 cells that were transiently transfected with pM1-HA, a vector that will express the HA tag alone. Due to the difference in size between the expressed HA-tag and the HA-tagged C/EBP proteins, the HA tag was run off the gel. As indicated in the control lane, the HA antibody did not interact with any non-specific proteins. The HA-tagged C/EBP proteins were expressed to comparable levels in HepG2 cells (with the exclusion of HA-αΔCR2) and the indicated proteins are the correct molecular weight. Other experiments have shown that HA-αΔCR2 is expressed to levels comparable to the other HA-tagged proteins. HA-αΔCR1,2 was not represented.

The activity of the PEPCK promoter (-490PCK) was induced by over-expression of PKA or HA-C/EBPα, and simultaneous over-expression of both proteins resulted in synergistic response (Figure 4.12). In Figure 4.12, all of the HA-tagged C/EBPα mutants had little, if any, constitutive activity but only HA-αΔCR1, HAαΔCR1,2 and HAαΔ-CR2 showed a reduction in PKA-inducible activity below that of the reporter

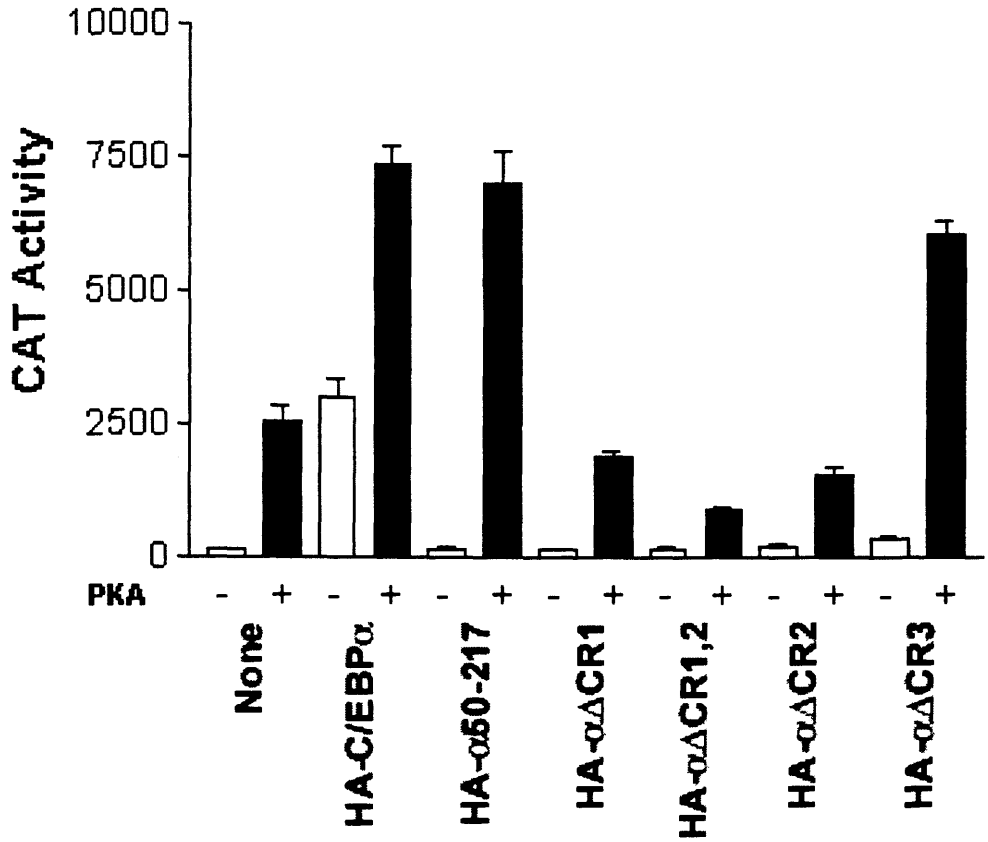


Figure 4.12: Effect of over-expression of C/EBPα mutants on the PKA-responsiveness of the PEPCK promoter. The reporter plasmid -490PCK (3.5 μg) was co-transfected into HepG2 cells in the absence (white bars) and presence (solid bars) of a PKA expression plasmid (1 μg) and 1 μg of each HA-tagged C/EBPα deletion construct. The control lane represents HepG2 cells that have not been transfected with a GAL4-C/EBPα expression plasmid. The values shown are the means +/- S.E. of at least three experiments.

gene alone. These data suggest that C/EBPα mutants which lack CR1 or CR2, inhibit the PKA-inducible activity of the reporter gene in a dominant negative fashion.

4.2.5 Effect of Constitutive and PKA-Inducible Activity of C/EBPα Deletion Mutants on the Glycoprotein α Subunit Promoter

The specificity of the dominant negative action of HA-αΔCR1 and HA-αΔCR2 were assessed by examining the effects of these proteins on the activity of the Glycoprotein α-subunit promoter. This promoter was selected because it is highly responsive to PKA and this responsiveness is mediated solely by two CREB proteins bound to two tandem consensus CRE sites (Silver *et al.* 1987). Moreover, C/EBPα does not bind well to these particular CREs (Roesler *et al.*, 1996). As shown in Figure 4.13, none of the indicated HA-C/EBPα proteins affected the constitutive activity or the PKA responsiveness of this promoter. These data suggest that the effect of C/EBPα deletion mutants is specific.

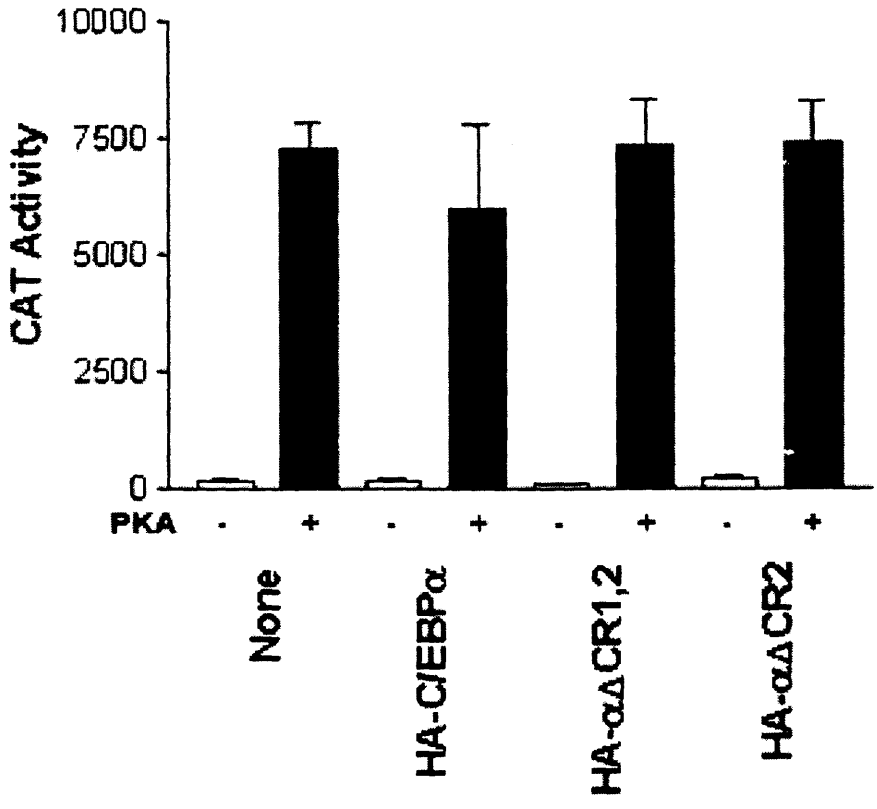


Figure 4.13: Effect of over-expression of C/EBPα mutants on the Glycoprotein α subunit promoter. The Glycoprotein α subunit-CAT promoter (3.5 μg) was cotransfected into HepG2 cells in the absence (solid bars) and presence (hatched bars) of a PKA expression plasmid (1 μg) and 1 μg of each HA-tagged C/EBPα deletion construct. The values shown are the means +/- S.E. of at least three experiments.

4.2.6 Effect of Promoter Environment on C/EBPα Activity

It is well documented that promoter environment can modify the precise activity that transcription factors exhibit (Fry and Farnham, 1999). The promoter used to assess the constitutive and PKA-inducible activities of the GAL4-C/EBPα mutants in Figures 4.9 and 4.10 had, in addition to GAL4 binding sites, an AP-1 binding site and a CRE, as these sites comprise parts of the CRU. To examine the influence that these sites might have on the activity of the GAL4 fusion proteins, the activities of the GAL4-C/EBPα mutants were tested on a promoter containing the first 68 bp of the PEPCK promoter (which contains only a TATA box) to which were linked four tandem GAL4 binding sites, termed -68GAL4*4 (Figure 4.14). The results shown in Figure 4.15 indicate that all of the mutants, with the exception of GAL4-αΔCR1,2, had decreased constitutive activity compared to GAL4-C/EBPα. Comparison of the deletion mutant data in Figure 4.15 (relative to GAL4-C/EBPα) with the deletion mutant data obtained in Figures 4.9 and 4.10 (relative to GAL4-C/EBPα) clearly indicate that the constitutive activity of the GAL4-C/EBPα fusion proteins was significantly modified by the promoter environment in which the mutants are assessed. For example, in Figure 4.10, GAL4-αΔCR1 and GAL4-αΔCR3, which lack CR1 and CR3, respectively, had constitutive

Figure 4.14: Schematic of the promoter, -68GAL4*4. The promoter –68GAL4*4 is composed of the first 68 bp of the PEPCK promoter, a minimal promoter consisting of a TATA box upstream of a CAT reporter gene, to which four tandem GAL4 binding sites are ligated.

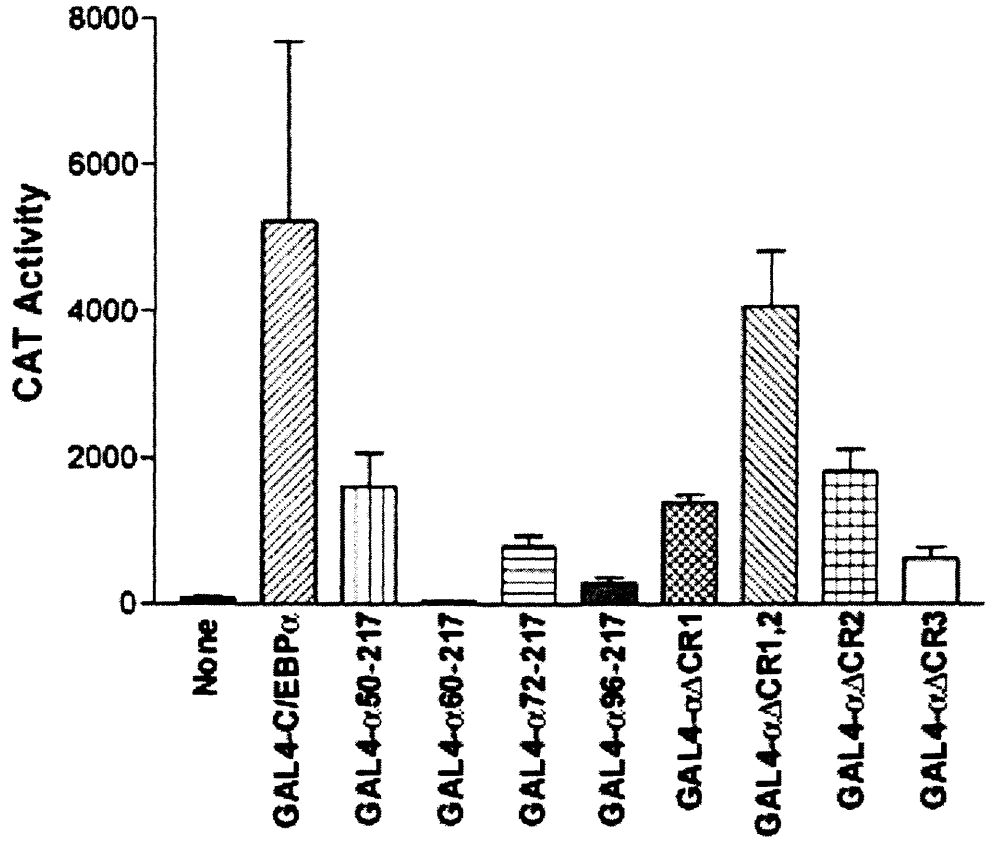


Figure 4.15: Constitutive activity of the GAL4-C/EBPα mutant proteins on –68GAL4*4. HepG2 cells were transiently co-transfected with 3.5 μg of -68GAL4*4 and 1 μg of each GAL4-C/EBPα fusion construct. Control lanes indicate HepG2 cells that have not been over-expressed with C/EBP expression plasmids. The values shown are the means +/- S.E. of at least three experiments.

activity comparable to GAL4-C/EBPα, yet in Figure 4.15, GAL4-αΔCR1 and GAL4-αΔCR3 had little constitutive activity compared to Gal4-C/EBPα. Also, in Figure 4.10, GAL4-αΔCR1,2 had poor constitutive activity compared to GAL4-C/EBPα but, in Figure 4.15, GAL4-αΔCR1,2 had constitutive activity comparable to GAL4-C/EBPα. In summary, the relative importance of specific domains within C/EBPα may vary depending on the nature of the promoter being studied.

4.2.7 Effect of Deletion Mutants on C/EBPα Activity When They are Tethered to PEPCK's CRE site

Based on the findings that promoter context may play an important role in determining the relative importance of specific domains within *C/EBPα, it was speculated that the domains within C/EBPα that mediate* the PKA-inducible response when it is bound to the sites within the LSR may be different when C/EBPα was tethered to the CRE. It is important to remember that although C/EBPα can occupy three sites within the LSR, it can also bind to the CRE site on the PEPCK promoter with affinity similar to that of CREB and C/EBPα has been shown to be able to fundamentally replace CREB at the CRE (Roesler, *et al.*, 1998). To test this hypothesis, transient transfection of the GAL4-linked C/EBPα wild type and deletion

mutants was performed in HepG2 cells using the artificial promoter G4PEPCK (Figure 4.16). This promoter is identical to the endogenous –490PCK vector except that the region corresponding to the CRE is replaced with a GAL4 site (Quinn, 1993). As shown in Figure 4.17, GAL4-C/EBPα, GAL4-αΔCR1 and GAL4-αΔCR2 mutants could mediate comparable constitutive activity. However, GAL4-α50-217 and GAL4-αΔCR3 mutants mediated a much poorer constitutive response compared to GAL4-C/EBPα. Similar results were observed for the effect these mutants had on the PKA-inducible activity when they were bound to the region corresponding to the CRE. GAL4-αΔCR1 and GAL4-αΔCR2 mutants could mediate a PKA-inducible response, albeit a lower response than that of GAL4-C/EBPα. However, GAL4-α50-217 and GAL4-αΔCR3 mutants mediated very little PKA-inducible activity compared to GAL4-C/EBPα when the proteins bound to the CRE. These data indicate that although CR1 and CR2 were absolutely required for C/EBPα to mediate the constitutive and PKA-inducible activities when it bound sites within the LSR (Figure 4.10), these same domains were not critical for the protein to mediate the PKA response when it was bound to the CRE. However, although GAL4-α50-217 and GAL4-αΔCR3 mediated a poor constitutive

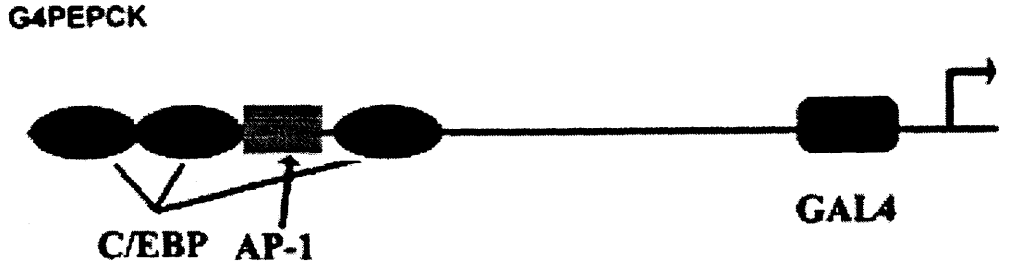


Figure 4.16: Schematic of the artificial PEPCK promoter, G4PEPCK. G4PEPCK consists of the native -490PCK promoter with the CRE site replaced with a GAL4 binding site.

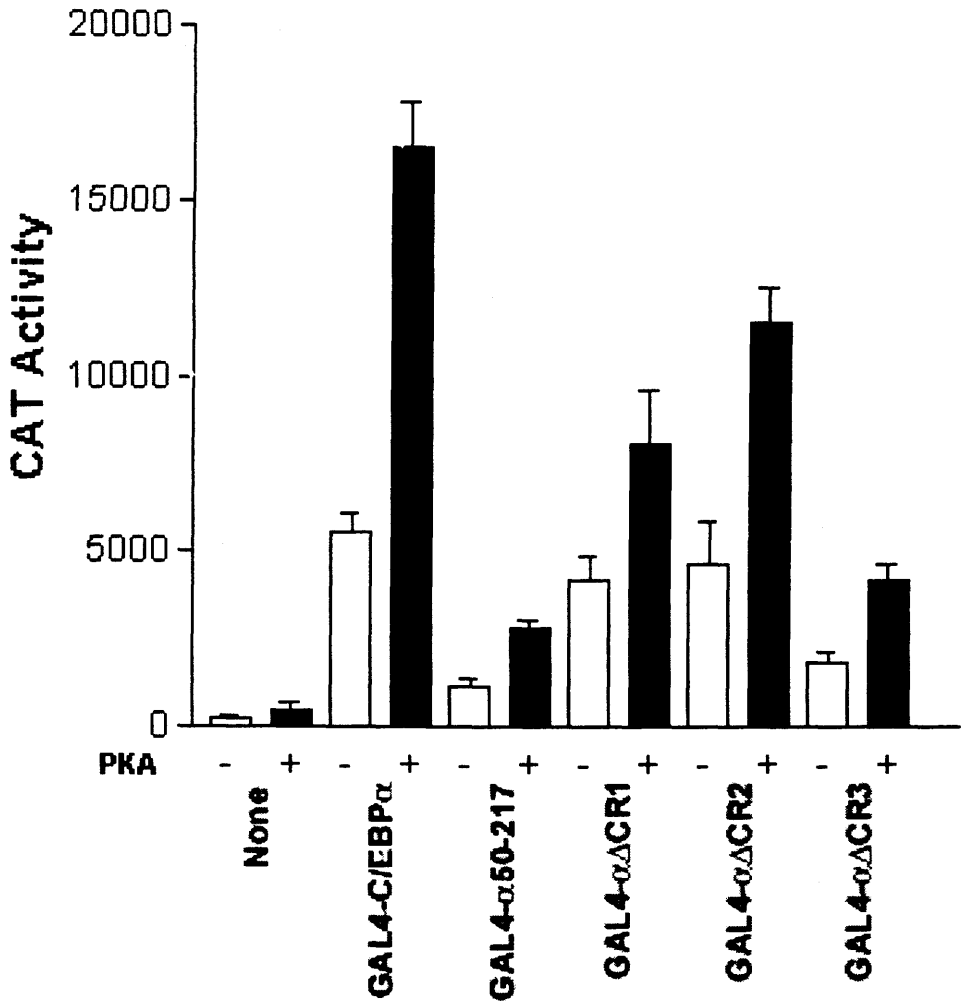


Figure 4.17: CR3 and the first 50 amino acids of C/EBPα mediate the PKA-inducible response when it binds to the CRE site on the PEPCK promoter. HepG2 cells were transiently transfected with 3.5 μg of the artificial reporter plasmid G4PEPCK in the absence (clear bars) and presence (solid bars) of 1 μg of the catalytic subunit of PKA and 1 μg of the GAL4-C/EBPα deletion constructs. Control lanes indicate HepG2 cells that were not transfected with GAL4-C/EBP expression plasmids. The values shown are the means +/- S.E. of at least three experiments.

and PKA-inducible activity when tethered to the CRE site (Figure 4.17), Figure 4.10 indicated that these regions were not critical to mediate the constitutive or PKA-inducible activity of the PEPCK gene when C/EBPα bound to the LSR. Together, these data indicate that not only can a transcription factor occupy multiple and variable sites on a promoter, but also that the site where a transcription factor binds to a promoter may dictate which domain(s) of the protein are used to mediate the constitutive and hormone-inducible response.

4.3 Characterization of Conserved Regions 1 and 2 in C/EBPβ

4.3.1 Rationale for Site-specific Deletion Analysis of C/EBPβ

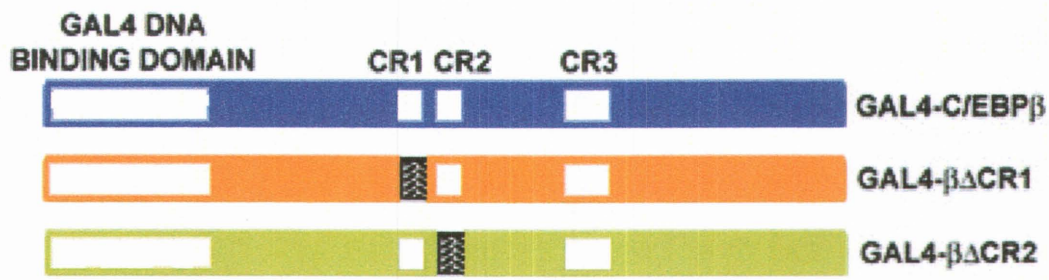
The next step in this analysis was to study the CRs within C/EBPβ to determine if they contribute to the constitutive or PKA-inducible responsiveness on the PEPCK promoter. Because CR3 is much less homologous than CR1 and CR2, only the latter were characterized in the C/EBPβ transcription factor. Figure 4.18A shows a schematic of the GAL4-C/EBPβ constructs. The regions corresponding to CR 1 and 2 of the C/EBPβ gene were independently excised from the parent construct and replaced with an oligonucleotide. Each oligonucleotide was designed so that, upon translation, the endogenous amino acids would be replaced

with alanines, ultimately disrupting the activity of the conserved region without altering the length of the protein.

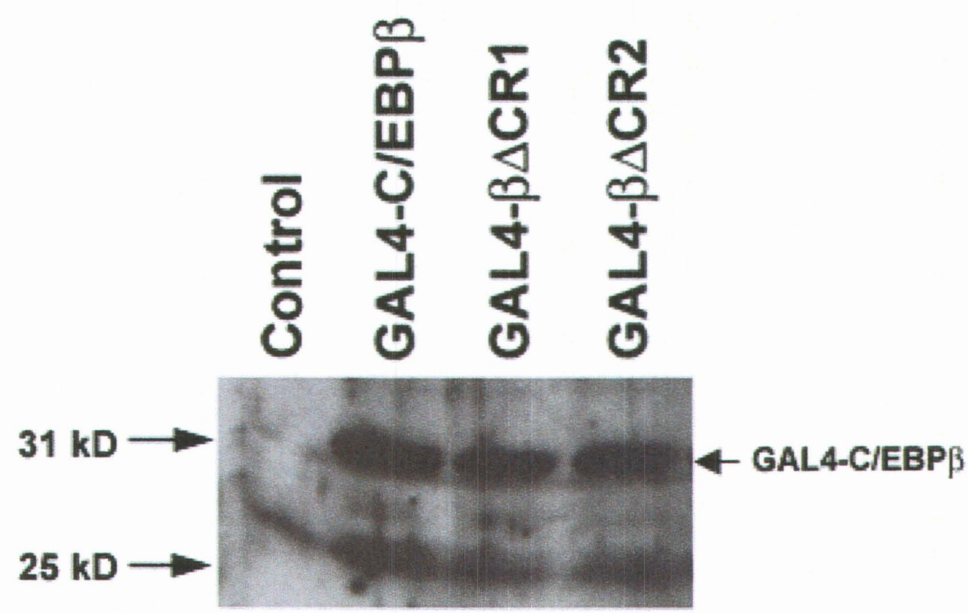
The GAL4 fusion protein expression levels were verified by Western analysis using a GAL4-specific antibody (Figure 4.18). Control lanes indicate HepG2 cells that were transiently transfected with the plasmid pM1 which expressed the GAL4 domain alone. Several non-specific bands were identified by comparison with the control lanes. In order to achieve sufficient separation of the protein bands, the GAL4-domain was run off the gel and thus did not show up in the controls lanes. GAL4-C/EBPβ (and the chimeras) were expressed to comparable levels in the HepG2 cells and they migrated to the predicted molecular weight.

Figure 4.18: Schematic and expression analysis of GAL4-C/EBPβ chimeras A) Schematic of the GAL4-C/EBPβ fusion proteins. GAL4-C/EBPβ contains the rat C/EBPβ N terminal transactivation domain (amino acids 1-108) linked to the DNA binding domain of the yeast transcription factor, GAL4. The remaining constructs depicted have modifications to the transactivation domain as shown but are otherwise similar to GAL4-C/EBPβ. B) Western analysis of GAL4-C/EBPβ deletion mutants. Transient transfection analysis was performed on HepG2 cells as indicated in Section 3.7.2 and the cells were processed as specified in Section 3.6. Equal concentration of proteins were electrophoresed on a 12% SDS-PAGE gel and transferred onto a nitrocellulose membrane. The mutants were detected with an antibody against the GAL4 DNA binding domain. Molecular weight markers are indicated.

A


GAL4 DNA
BINDING DOMAIN
CR1 CR2
CR3
GAL4-C/EBPβ
GAL4-βΔCR1
GAL4-βΔCR2

B


Control
GAL4-C/EBPβ
GAL4-βΔCR1
GAL4-βΔCR2
31 kD
25 kD
GAL4-C/EBPβ

4.3.2 Effects of Promoter Environment on C/EBPβ Mutant Construct Activity

The GAL4-C/EBPβ mutants were assessed for their constitutive and PKA-inducible activities in the presence of the permissive promoters G4PEPCK, -109G3/A1 and –68GAL4*4 (Figure 4.19). Unlike the C/EBPα CR mutants, mutation of CR1 and CR2 of C/EBPβ did not appear to affect the PKA-inducible response on any of the three PEPCK promoters. The constitutive activity of the CR2 mutant was reduced when tested on the -68GAL4*4 promoter but the activity of the mutant was strong relative to GAL4-C/EBPβ when expressed in the presence of the other two promoters. These data indicate that the CR1 and CR2 in C/EBPβ are not independently responsible for mediating the constitutive or PKA-inducible activity regardless of which *cis*-elements C/EBPβ occupied on the PEPCK promoter.

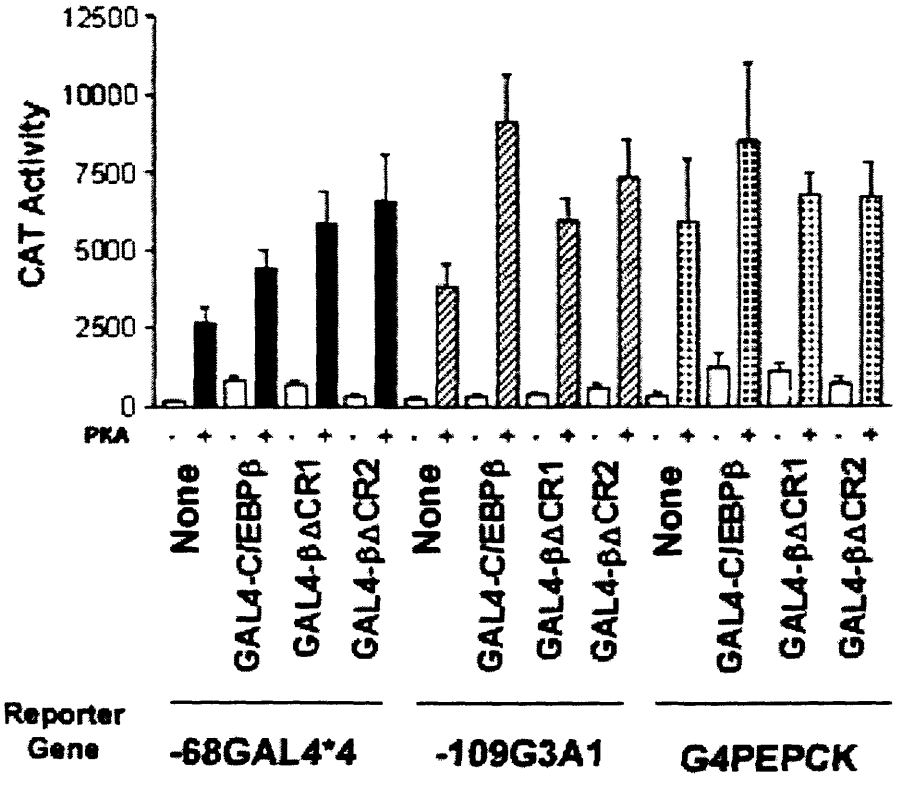


Figure 4.19: The conserved regions in C/EBPβ do not mediate PKA-inducible activity. HepG2 cells were transiently transfected with 3.5 μg of each respective promoter in the absence (clear bars) and presence (hatched bars) of a PKA expression vector (1 μg) and 1 μg of each GAL4-C/EBPβ mutant construct, as indicated. The control lanes indicate HepG2 cells that have not been transfected with GAL4-expression plasmids. The values shown are the means +/- S.E. of at least three experiments.

4.4 Protein-Protein Interaction Between C/EBPα and TBP and TFIIB

4.4.1 Rationale for Investigating a Physical Interaction Between C/EBPα chimeras and GST-TBP and GST-TFIIB

Nerlov and Ziff (1995) identified regions within C/EBPα that were critical for mediating constitutive activity. They also performed GST-pull down assays that indicated that amino acids 1-96 were responsible for mediating an interaction with TBP and TFIIB, members of the general transcription apparatus. Nerlov and Ziff further refined the region of the C/EBPα transactivation domain that is necessary to mediate the constitutive activity by selective amino acid mutation analysis. They created a C/EBPα mutant with a triple mutation (amino acids 67, 77 and 78 were converted from tyrosine, phenylalanine and leucine, respectively, to alanines) and studied this triple mutant (C/EBPα TM) in HepG2 cell transient transfection analysis (Nerlov and Ziff, 1995). Construction of C/EBPα TM resulted in a mutant that failed to mediate a constitutive response. These results were substantiated by Roesler, *et al.* (1998) who showed that C/EBPα TM failed to mediate a constitutive response yet it was able to mediate a substantive PKA-inducible response on the PEPCK promoter. The C/EBPα deletion mutants described in this thesis and the

C/EBPα TM were *in vitro* transcribed and translated to determine if these chimeras could physically interact with GST-TBP and/or GST-TFIIB.

4.4.2 GST-Pull Down Assay With C/EBPα chimeras and GST-TBP and GST–TFIIB

The INPUT panel indicates each ^{35}S-labeled protein was expressed using a coupled *in vitro* transcription/translation kit. The darkest band in each input lane represents the slowest migrating protein and these proteins reside at a position on the gel that accurately reflects its predicted molecular weight. Faster migrating proteins in each lane may represent incomplete translation products and or proteins that have been translated from an alternate AUG initiation codon.

GST pull-down analysis indicated that radiolabeled ^{35}S-C/EBPα physically interacted with both GST-TBP and GST-TFIIB (Figure 4.20). Removal of the first 50 amino acids (^{35}S-α50-217) or deletion of CR3 (^{35}S-αΔCR3) resulted in a protein that failed to interact with GST-TBP or GST-TFIIB. However, ^{35}S-αΔCR1 and ^{35}S-αΔCR2 did retain the ability to

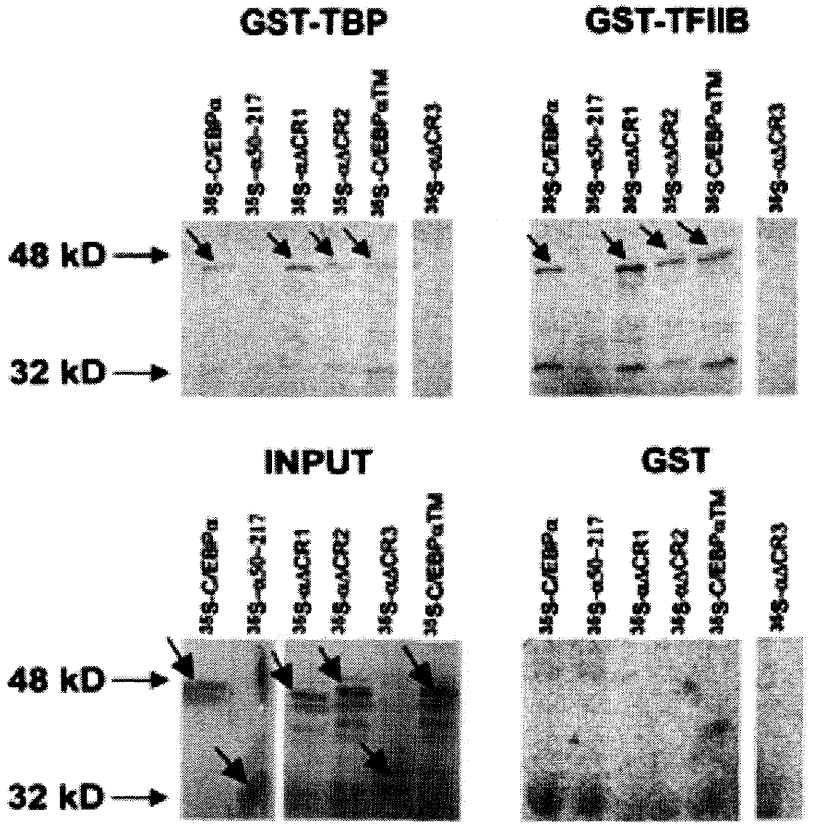


Figure 4.20: CR3 and the first 50 amino acids of C/EBPα are required to physically interact with GST-TBP and GST-TFIIB. GST-tag panels: A volume of 8 μL of ^{35}S-labeled C/EBPα chimeras (expressed using Promega's TNT Transcription/Translation kit) were introduced to 10 μg of GST-fusion proteins or GST alone bound to 100 μL of glutathione-Sepharose. INPUT panel: 20% of the radiolabeled protein incubated with the GST proteins were loaded into a 12 % SDS-PAGE gel. The bands were detected by autoradiography. Molecular weight markers are indicated. The predicted M.W. of each proteins area as follows: ^{35}S-C/EBPα = 42 kDa; ^{35}S-α50-217=36.5 kDa; ^{35}S-αΔCR1=41 kDa; ^{35}S-αΔCR2=41 kDa; ^{35}S-αΔCR3= 38 kDa; ^{35}S-C/EBPα TM= 42 kDa.

interact with TFIIB and TBP (Figure 4.20). The ability of the C/EBPα TM to interact with TBP and TFIIB was surprising given that transient transfection analysis showed C/EBPα TM failed to mediate constitutive activity on the PEPCK promoter (Nerlov and Ziff, 1995; Roesler, *et al.* 1998). None of the ^{35}S-labeled mutant C/EBPα proteins interacted with GST, as indicated by the absence of bands in the GST panel.

5. DISCUSSION

5.1 Role of Conserved Regions Within C/EBPα in Mediating the Constitutive and PKA-Inducible Response on the Hepatic PEPCK Promoter

Structure/function analyses of C/EBPα and C/EBPβ have indicated that their constitutive transactivation domain(s) reside within the N-terminal portion of the protein, while the DNA-binding motif contributes little if any to the constitutive transactivation function of the protein (Friedman and McKnight, 1990; Nerlov and Ziff, 1994; Pei and Shih, 1991; Roesler, *et al.*, 1996). Previous studies have also indicated that the PKA-inducible domain of C/EBPα resides within residues 50-124 (Roesler *et al.*, 1998), while the PKA-inducible activity of C/EBPβ is mediated by residues 1-108, although residues 1-66 show significant activity (Park *et al.*, 1999). The data in this thesis indicate that CR2 plays a critical role in mediating the PKA-inducible activity of C/EBPα when it occupies sites corresponding to the LSR. This conclusion is supported by the observations that (1) a GAL4-C/EBPα fusion protein containing residues

50-217 retained PKA-inducible activity whereas a fusion protein containing residues 60-217, which lacks CR1 and CR2, lost this activity (Figure 4.9), (2) a GAL4-C/EBPα fusion protein containing an internal deletion of CR2 was unable to mediate the PKA responsiveness when it was tethered to the distal sites on the promoter (Figure 4.10), and (3) the loss of PKA-inducible activity due to the CR2 deletion does not appear to be simply a consequence of a generalized loss of activity, since this mutant retains the ability to confer a significant level of constitutive expression (Figures 4.15). Because CR2 appears to be critical for mediating C/EBPα's PKA-inducible activity when the protein binds to the distal CRU sites on the PEPCK promoter, over-expression of a full-length version of C/EBPα with a deletion in CR2 was evaluated to determine if this mutant inhibits the PKA responsiveness of the PEPCK promoter due to a dominant-negative type of mechanism. Figure 4.12 shows that the PEPCK promoter (-490PCK) is robustly responsive to over-expression of PKA or HA-C/EBPα, and simultaneous over-expression of both resulted in a synergistic response. A substantial loss of PKA responsiveness was observed in the presence of HA-αΔCR1,2 and HA-αΔCR2 (Figure 4.12) which suggests that these mutants are acting in a dominant negative fashion.

The effects of the other GAL4-mutants indicate that GAL4-α50-217, GAL4-αΔCR1 and GAL4-αΔCR3 proteins mediate a PKA-inducible response that was approximately 50% of that seen with the full length C/EBPα transactivation domain (Figure 4.9, Figure 4.10). While one could argue that these deletions might cause non-specific tertiary structure alterations in the protein, the observation that the specified mutants retained significant constitutive transactivation activity (Figure 4.9, Figure 4.10) indicated that no gross structural changes occurred as a result of these mutations. Over-expression of HA-α50-217, HA-αΔCR1 and HA-αΔCR3 deletion mutants resulted in little constitutive activation of the PEPCK promoter although HA-α50-217 and HA-αΔCR3 did mediate PKA-inducible activity to levels comparable to the wild type protein (Figure 4.12). Like HA-αΔCR2, HA-αΔCR1 inhibited the PKA-inducible activity of PEPCK in a dominant negative fashion. Although other regions within C/EBPα contribute to the PKA-inducible response on the PEPCK promoter, only CR2 appears to be absolutely required to mediate this PKA-inducible effect.

5.1.1 The Constitutive and PKA-Inducible Domains of C/EBPα Are Distinct But They May Over-Lap to a Degree

Several pieces of evidence suggest that the mechanism(s) whereby C/EBPα mediates constitutive transactivation and PKA-inducible transactivation are different, and involve distinct albeit overlapping domains and/or amino acid residues. First, a GAL4-C/EBPα fusion protein containing the entire N-terminal transactivation domain lacks any detectable constitutive activity when examined in the choriocarcinoma JEG-3 cell line, yet it maintains PKA-inducible activity (Roesler *et al.*, 1996). Second, studies with the C/EBPα TM show that this mutant fails to mediate a constitutive response although it retains its PKA-inducible activity. The data present in this thesis suggesting that CR2 is critical for mediating the PKA-inducible activity when C/EBPα binds to the LSR, are supported by the triple mutant data described above. Two of the three mutations in C/EBPα TM (positions 77 and 78) lie outside of CR2, which spans residues 62-70 (Figure 4.5). The third mutation in the triple mutant (residue 67) lies within CR2. Not surprisingly, this point mutation was shown by Nerlov and Ziff (1995) to have little independent effect on the constitutive activity of C/EBPα, but required the additional mutations at amino acids 77 and 78 for its effect to be noticed. Third, the C/EBPα

deletion mutant GAL4-αΔCR3 has a diminished PKA inducible activity yet it maintains its constitutive activity when it binds to the LSR (Figure 4.10). Taken together, these data suggest that the C/EBPα domains that mediate its constitutive and PKA-inducible activities and the mechanisms whereby this protein mediates these activities are distinct, although some amino acids/domains of the protein may participate in both responses.

5.1.2 Promoter Environment Affects Transcription Factor Activity

The data presented in this thesis suggest that promoter context may influence the specific domain in a transcription factor that is used to mediate transactivation. There is evidence that a transcription factor may bind to conserved *cis*-elements on two promoters in a structurally distinct manner. The pituitary gland consists of six hormone-secreting cell types, three of which expresses the transcription factor Pit-1 which induces expression of the growth hormone, prolactin, and thyroid stimulating genes in somatotrope, lactotrope, and thyrotrope cell types, respectively (Pfaffle, *et al.*, 1996). Pit-1 is a pituitary-specific, POU domain transcription factor that activates growth hormone gene expression in somatotropes, yet it restricts growth hormone gene expression in lactotropes (Scully, *et al.*, 2000). The prolactin gene promoter has a Pit-1

site called the Prl-1P element that is conserved with the GH-1 element on the growth hormone gene promoter (Scully, *et al.*, 2000). Therefore, although Pit-1 binds the prolactin gene promoter to induce expression, Pit-1 binds to a conserved site on the growth hormone gene promoter to repress its expression within the same cell line. Crystallography studies have revealed a striking structural difference in how the bipartite POU DNA binding domain within Pit-1 is accommodated on these two promoters. The spacing between the DNA contacts made by Pit-1 changed from 4 bp upon binding to the *cis*-element in the Prl-1P element to 6 bp upon binding the GH-1 element (Scully, *et al.*, 2000). Overall, protein-DNA contacts in the major groove were generally similar between the two complexes. The GH-1 complex, however, lacked minor groove contacts because the Pit-1 terminal arm did not penetrate the minor groove to the same extent as in the Prl-1P complex (Scully, *et al.*, 2000). Ultimately, Pit-1 binding to the growth hormone gene promoter promoted recruitment of a co-repressor complex whereas Pit-1 binding to the prolactin gene promoter activated gene expression in lactotropes.

The PEPCK promoter is complex; it is composed of multiple protein binding sites to which a number of different transcription factors can bind. Therefore, each *cis*-element is present within a unique

"microenvironment" which may specifically affect the activity of a transcription factor. Analysis of a chimeric transcription factor on a synthetic promoter may give valuable information about the potential of a transcription factor to mediate a constitutive or hormone inducible response, but it does not take into account the ability of the protein to regulate expression on a natural promoter and it may in fact lead to misinterpretation of the role of the protein in the context of the natural promoter (Fry and Farnham, 1999). The ability of promoter-context to regulate transcription by modifying the precise activity of a transcription factor may involve transcription factor-induced DNA bending, overlapping binding sites for transcription factors, protein-protein interactions, the structure of the core promoter, and the particular cellular environment (Fry and Farnham, 1999). Comparison of data obtained using –109/G3A1 (Figures 4.9 and 4.10) and –68GAL4*4 (Figure 4.15) clearly indicate that the constitutive activity of the GAL4-C/EBPα fusion proteins are significantly modified by promoter environment and these data argue strongly that analysis of both *cis*-elements and the corresponding transcription factor should be performed in the context of the natural promoter.

5.1.2.1 Where C/EBPα Binds to the PEPCK Promoter May Dictate Which Domains Mediate its PKA-Inducible Response

In vitro binding assays have indicated that more than one transcription factor can bind to most if not all of the *cis*-elements involved in the PEPCK promoter CRU with similar affinity. The proteins that can bind to the CRE include CREB (Park, *et al.*, 1990), C/EBPα (Roesler, *et al.*, 1993) C/EBPβ (Yamada, et al., 1999), some ATFs (Rehfuss, *et al.*, 1991), D-site protein (Roesler, *et al.*, 1992), and AP-1 related proteins (Gurney, *et al.*, 1992). C/EBPα (Park, *et al.*, 1990), C/EBPβ (Liu, *et al.*, 1991) and D-site binding protein (Gurney, *et al.*, 1992) can bind to the three distal C/EBP binding sites in the promoter, *in vitro*. Different levels of PKA responsivity may be achieved with different patterns of transcription factor binding; a mechanism that has been proposed to explain the benefits of hormone response units (Roesler and Park, 1998).

When C/EBPα is functioning through the LSR on the PEPCK promoter, CR2 is critical for mediating the PKA-inducible activity (Figure 4.10, 5.1). When binding to the LSR, GAL4-α50-217 and GAL4-αΔCR3 do not appear to be critical for mediating the constitutive or PKA-inducible activity although these mutants do mediate a reduced level of reporter gene expression in the presence of PKA compared to the GAL4-C/EBPα.

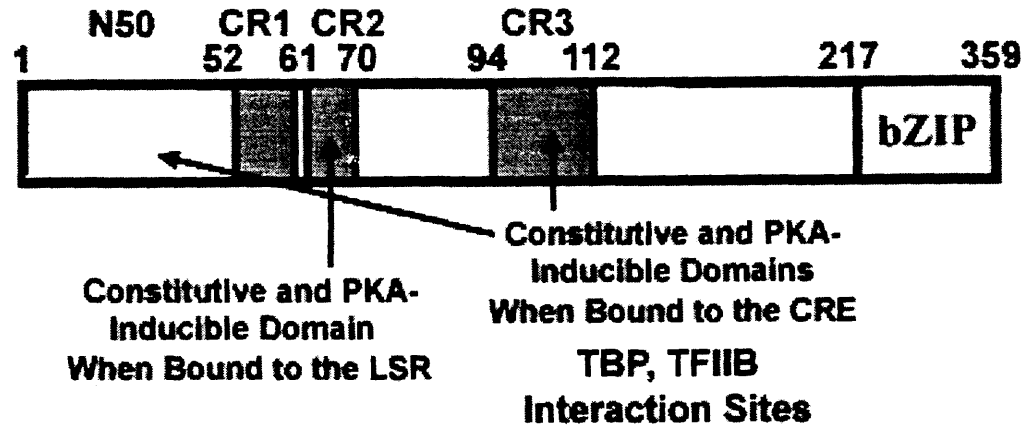
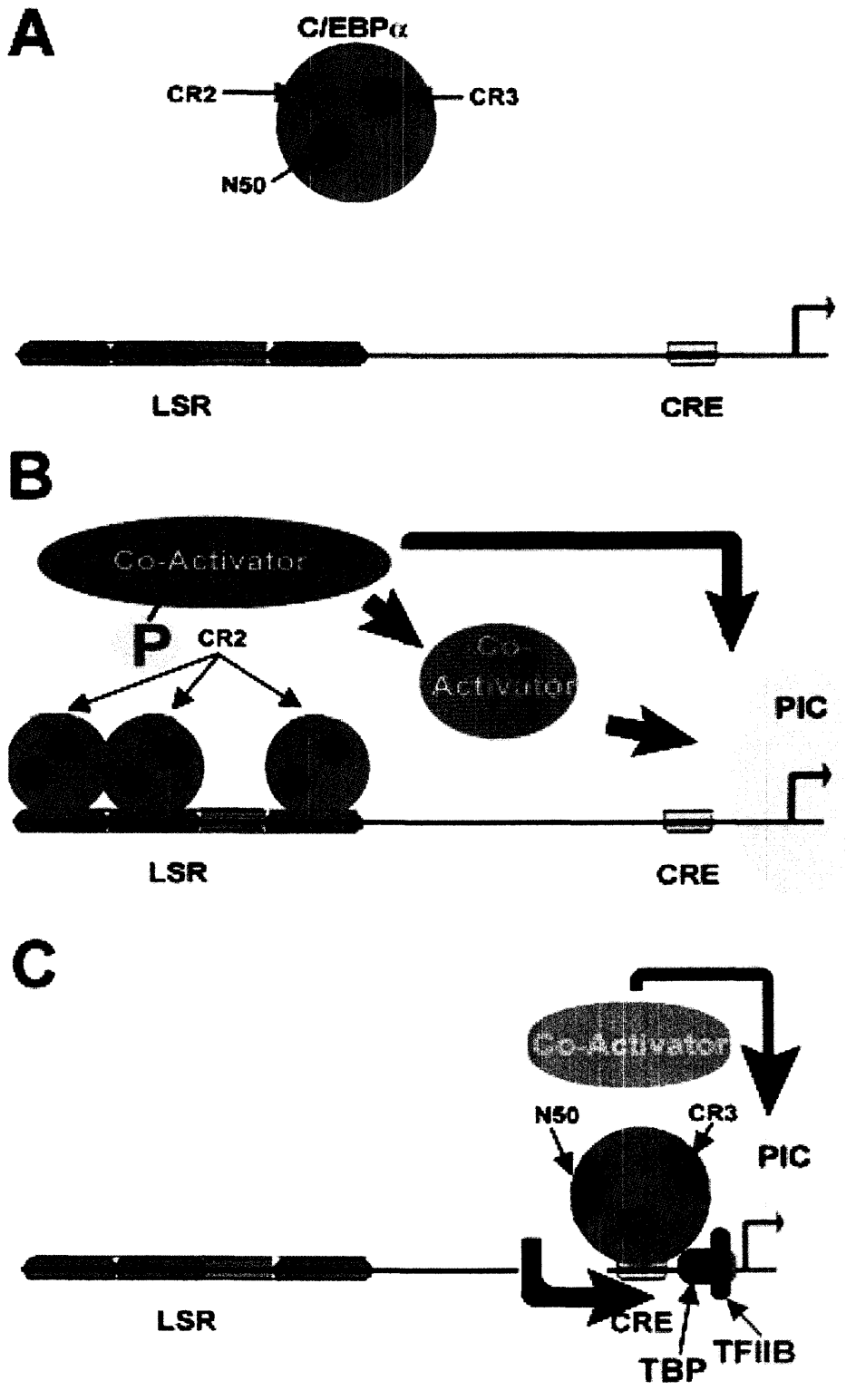


Figure 5.1: Schematic of conserved regions within C/EBPα protein responsible for mediating its constitutive and PKA-inducible responses. The numbers indicate the amino acid residues on the protein. The domains indicating the conserved regions of C/EBPα are shaded gray; the amino acids 1-52 are indicated as N50. The region within C/EBPα that mediates protein-protein interactions with TATA binding protein (TBP) and TATA binding protein associated factor II B (TFIIB) are indicated. The basic region-leucine zipper DNA-binding motif resides at the carboxy-terminus of the protein and does not appear to contribute to its constitutive or PKA-inducible activity.

However, when GAL4-C/EBPα is bound to the CRE region, removal of the first 50 amino acids or CR3, and not CR2, greatly reduces both the constitutive and PKA inducible activity of the protein (Figure 4.17, 5.1). These data highlight a new level of complexity regarding the regulation of transcription initiation. The domain within the C/EBP protein that is responsible for mediating the constitutive or PKA responsiveness differs depending on which *cis*-element(s) it occupies on the PEPCK promoter (Figures. 4.10, 4.17, 5.1).

There are multiple *cis*-elements adjacent to the LSR and the CRE that do not directly contribute to the PKA response of the promoter. However, in the presence of PKA, distinct co-activators and/or transcription factors may be recruited to the promoter due to the complimentary protein contact surfaces within the enhancesome. It may be that the conformational structure of C/EBPα is remodeled by adjacent factors depending on which DNA element it binds and/or depending on which proteins it interacts with on the promoter. Transcription factor synergism may arise due to changes in each protein's ability to bind to DNA and specific protein/protein interaction may allow the enhancesome or a specific protein to interact with members of the general transcription apparatus (Herschlag and Johnson, 1993). Clearly there is the opportunity

for unique protein-protein interactions to occur between C/EBPα and other proteins, and these interactions may cause conformational changes that "unmask" or make more prominent a specific domain of C/EBPα that can be used to mediate transactivation. The presentation of different domains might, for example, result in the recruitment of different co-activators and/or contacts with different general transcription factors that may confer a unique transcriptional response. Nerlov and Ziff (1995) performed GST-pull down assays with non-overlapping portions of C/EBPα's transactivation domain and GST-TBP and GST–TFIIB. These studies indicated that the N terminus of C/EBPα's transactivation domain is critical for interaction with these general transcription factors. The data in this thesis, however, indicate that although the extreme N terminus of the transactivation domain is critical for interaction with TBP and TFIIB, the region corresponding to CR3 is also required for interaction with these GST-fusions (Figure 4.23). CR2, the region of C/EBPα that is critical for mediating the PKA-inducible activity when it occupies sites on the LSR, is not required to physically interact with TBP or TFIIB. These data may explain the relative importance of the different domains of C/EBPα when it binds to different sites on the PEPCK promoter (Figure 5.2). When C/EBPα is bound to PEPCK's CRE site, the N terminal 50 amino acids and


A
C/EBPα
CR2
CR3
N50
LSR
CRE
B
Co-Activator
P
CR2
Co-Activator
PIC
LSR
CRE
C
Co-Activator
N50
CR3
PIC
LSR
CRE
TBP
TFIIB

CR3 may mediate the constitutive and/or PKA-inducible activity by physical interaction with members of the preinitiation complex. However, when C/EBPα binds to the LSR, the conformational shape of the protein may be such that these domains cannot interact with TBP or TFIIB. Instead, the tertiary structure of C/EBPα may allow contact with co-activators (possibly through CR2) that bridge C/EBPα to the preinitiation complex. The domains within a transcription factor that mediate a constitutive or hormone inducible response may be made accessible (or in-accessible) to potential interactors depending on which *cis*-element it occupies on the promoter.

5.2 Characterization of the Constitutive and PKA-Inducible Activity of C/EBPβ

It was previously thought that, unlike C/EBPα, the C/EBPβ isoform could not mediate a PKA response and it would, in fact, inhibit the PKA response of the PEPCK reporter gene (Roesler, *et al.*, 1998). Park, *et al.*, (1993) came to a similar conclusion when testing the effect of PKA on activation of the PEPCK promoter in the presence of the expression plasmids for full-length C/EBPα, C/EBPβ and CREB. They used the CAT reporter gene –134 PEPCK-CAT. This region of the PEPCK promoter

contains the natural CRE site and TATA box but not the LSR. Unlike C/EBPα or CREB, over-expression of C/EBPβ in the presence of PKA did not augment transcription of the reporter gene (Park, *et al.*, 1993). The data presented by Park, *et al.*, (1999) indicated, however, that once a significant portion of the transactivation domain and the entire bZIP domain of C/EBPβ is replaced with an exogenous DNA binding motif, this chimera can mediate a PKA-inducible response on the PEPCK promoter when C/EBPβ$_{1\text{-}108}$ occupies the CRE (Park, *et al.*, 1999). Two possible conclusions from these studies could be that 1) a putative attenuation domain resides within C/EBPβ between amino acids 108 and its bZIP domain or 2) full-length C/EBPβ cannot physiologically induce transcription of the PEPCK gene when it is bound to the CRE unless its activity is "unmasked" artificially or by a radical conformational change in the protein induced by post-translational modification.

To determine whether there is a potential attenuation domain within C/EBPβ, the bZIP domains of C/EBPα and C/EBPβ were exchanged and the effect of these mutants were tested by transient transfection analysis in HepG2 cells. Unlike C/EBPβ, over-expression of wild type C/EBPα enhanced the PKA responsiveness of the PEPCK promoter (Figure 4.2). Over-expression of the two swapped mutants, C/EBPαβ and

C/EBPβα, could mediate a significant PKA response suggesting that the DNA binding domain of C/EBPα does not exert an inhibitory effect on the transactivation domain of C/EBPβ nor does the DNA binding domain of C/EBPβ negatively effect the transactivation potential of C/EBPα. It appears that even though the bZIP domains of C/EBPα and C/EBPβ are 71% identical and 95% conserved, the bZIP domain of C/EBPβ exerts a negative effect specific to it own transactivation domain.

Lee *et al.*, (1997b) discovered that C/EBPβ chimeras fused to the leucine zipper domain of C/EBPα failed to induce liver specific P-450 expression. Although these two isoforms share extensive homology within their leucine zipper domains, they suggested that this discrepancy in function may arise due to the ability of the C/EBPβ leucine zipper to better dimerize with an as yet unidentified bZIP protein. While studying the effect of these leucine zipper "swap" chimeras on the expression of the PEPCK reporter gene in HepG2 cells, it was identified that the C/EBPαβLZ chimera produced a transcription factor that induced the PKA response to a greater degree than the wild type C/EBPα protein yet the C/EBPβαLZ chimera mediated a lesser PKA-inducible response than the wild type C/EBPβ protein (Figure 4.5). It is possible that the leucine zipper domain of C/EBPβ lends itself to the formation of more stable

dimers. Therefore, although C/EBPα can mediate a stronger transactivation potential in the presence of PKA than C/EBPβ, a chimera of C/EBPαβLZ may form more stable dimers than the C/EBPα wild type protein resulting in a protein that can mediate a stronger PKA-inducible response.

Although CR2 of C/EBPα appears to be critical for mediating the PKA-inducible activity when it occupies the LSR and the first 50 amino acids and CR3 contribute to the constitutive and PKA-inducible activity *when it binds the CRE on the PEPCK promoter, the conserved regions* within C/EBPβ do not appear to contribute to the constitutive or PKA-responsiveness on the PEPCK promoter (Figure 4.20). Although the regions responsible for mediating C/EBPβ's PKA-inducible response may lie within the first 108 amino acids of its transactivation domain, the variable tertiary structure of the protein may bring together distant amino acids to comprise the PKA responsive region. Thus, although C/EBPα and C/EBPβ share some degree of homology within regions of each protein that mediate a significant PKA-inducible response, the term "conserved region" may not extend to a "conserved function".

The transcription factors C/EBPα and C/EBPβ$_{1\text{-}108}$ are functionally interchangeable within the GAL4 context when they occupy the LSR or

the CRE of the PEPCK promoter (Park *et al.*, 1999; Wilson *et al.*, submitted) and, in a recent review, it was suggested that, like CREB, C/EBPα and C/EBPβ have an endogenous PKA-inducible domain (Wilson and Roesler, 2002). Because a region within C/EBPβ can be removed resulting in the "restoration" of its ability to mediate a PKA-inducible response, it may be that although this protein is capable of mediating a PKA response, this response may not be realized by C/EBPβ simply binding to the *cis*-elements within PEPCK's LSR or CRE. It may be that, within the cellular milieu, C/EBPβ, unlike C/EBPα, must be phosphorylated or otherwise modified to alter the conformational shape of its tertiary structure, "unmasking" its PKA-inducible activity. C/EBPβ can be phosphorylated on multiple sites within its transactivation domain and DNA binding domain by a variety of kinases including PKA and PKC (reviewed in Roesler, 2001). For example, studies by Trautwein *et al.*, (1994; 1995) indicate that Ser^{240} of rat C/EBPβ can be phosphorylated by PKA and PKC *in vitro* and, upon phosphorylation, C/EBPβ cannot bind DNA. However, other studies indicate that C/EBPβ is not phosphorylated by PKA to any great extent (Wegner *et al.*, 1992). Thus, some uncertainty remains regarding the role of kinases in regulating the intrinsic activities of this C/EBP isoform. It may be that one or more kinase is called upon to

phosphorylate C/EBPβ and "unmask" the PKA-inducible activity on the PEPCK promoter.

5.3 Recruitment of Specific C/EBP Family Members to *cis*-Elements on a Promoter

When studying transcriptional activation, it is important to consider how members of a family that share considerable homology within their DNA binding domains, such that they display similar or identical DNA binding specificities, can differentially regulate sets of target genes. C/EBPα, C/EBPβ and CREB bind with near equal affinity to the CRE site and the C/EBP isoforms bind with similar affinity to the sites on the LSR on the PEPCK promoter (Park *et al.*, 1990; 1993). However, although multiple proteins can bind to the *cis*-elements on the PEPCK promoter, there may be specific recruitment of distinct proteins and/or family members to specific *cis*-element on the promoter in the presence of specific hormones. For example, because C/EBPβ, and not C/EBPα nor CREB, mediate the glucocorticoid response when it occupies the CRE site on the PEPCK promoter, C/EBPβ may be recruited to the CRE site to the exclusion of C/EBPα and CREB in the presence of glucocorticoids (Yamada, *et al.*, 1999). DNA binding affinity may not account for the

specific recruitment of one transcription factor over another on the PEPCK promoter in the presence of specific hormones.

The explanation of how each transcription factor is recruited to the promoter may lie within the creation of hormone-specific enhancesomes. In the presence of each hormone, distinct co-activators and/or transcription factors may be recruited to the promoter. The recruitment of these proteins may simply arise due to the *"jigsaw puzzle"* concept of complementary protein contact surfaces within the enhancesome (McKnight, 2001). One protein or a specific protein family isoform may be recruited to a site not due to the effects of protein kinases or DNA binding affinities but due to the tertiary structure of the protein being able to "fit" within the complex combination of other adjacent proteins. Evidence is accumulating in the literature that suggests that transcription factors synergize by aiding protein/DNA interaction or aiding in protein binding within the enhancesome. Lee *et al.*, (1997b) suggest that the reason why C/EBPβ, but not C/EBPα, is recruited to P-450 gene promoter is due to the different abilities of these two proteins to interact with adjacent proteins on the promoter. With respect to the CRU of the PEPCK promoter, the synergism observed when all five sites of the CRU are occupied may arise due to the combination of transcription factors allowing one or more

inducible domains of C/EBPs lack a PKA phosphorylation site, although C/EBPβ may be phosphorylated within its bZIP domain which may in turn affect its DNA-binding activity (reviewed in Roesler, 2001). Thus, the mechanism whereby these domains in C/EBPs are "activated" by PKA is unclear. There may be a co-activator that may interact with the PKA-inducible domains of C/EBP only after itself being phosphorylated by PKA. This scenario is suggestive of a model similar to that established for the mechanism of CREB activation, whereby CREB is phosphorylated by PKA which then allows recruitment of the co-activator CBP, except that the phosphorylation of the co-activator rather than the DNA-binding protein would regulate the interaction. Indeed, there is recent evidence that phosphorylation of a co-activator can selectively modulate the transcription factor with which it interacts (Yang *et al.*, 2001). This intrinsic activity may only manifest itself within certain promoter contexts due to the involvement of other so-called "accessory factors". CBP/p300 mediates the PKA-inducible activity of CREB, and has been shown to be a possible co-activator of C/EBPs (Erickson *et al.*, 2001; Mink *et al.*, 1997) however, there is no direct evidence in the literature to support this hypothesis. Since C/EBPs mediate the PKA response of the PEPCK promoter as components of a complex CRU, it is possible that co-activator

recruitment is achieved only by the coordinated action of several transcription factors. This hypothesis may explain the inability to observe some protein-protein interactions between co-activators and transcription factors even though a functional interaction can be detected. Clearly, identification, isolation and characterization of co-activators of C/EBPα and C/EBPβ are required in order to elucidate how these transcription factors exert their diverse effects on gene expression.

5.5 Future Directions

The future direction of this work must determine whether C/EBPα conforms to a specific and distinct tertiary structure depending on which *cis*-element(s) it occupies on the PEPCK promoter. How C/EBPα mediates its differential activity may not be dependent upon structure at all; it may mediate its activity by interacting with distinct proteins within the enhancesome. In other words, the microenvironment of the promoter may dictate which isoform of C/EBP binds to the PEPCK promoter and/or which domains within the protein mediate the constitutive and PKA-inducible activity. To this end, information must be gathered about which coactivators interact with C/EBPα when it binds to the CRE site and/or the LSR *in vivo* in the presence and absence of hormones. Similarly, when

C/EBPα binds to the LSR or the CRE, the identity of adjacent transcription factors may help to explain why a specific isoform and/or domain of C/EBP mediates the constitutive and PKA-inducible activity. Gaining greater information about the microenvironment of the PEPCK promoter and hormone specific enhancesomes may aid our overall understanding of eukaryotic transcriptional regulation.

5.6 Concluding Remarks

The data in this thesis indicate that the relative importance of the various conserved regions within C/EBP proteins may be dependent upon which *cis*-element it occupies on the PEPCK promoter. The "microenvironment" of each region of the promoter may dictate the overall conformational shape of the protein. Protein binding to adjacent sites on a gene promoter may directly or indirectly influence the binding ability or conformational shape of a transcription factor such that the region of the protein that is important for mediating its constitutive and/or PKA-inducible response is influenced by where it binds to the promoter. When C/EBPα binds to the CRE site on the PEPCK promoter, its tertiary structure may be such that the domains that interact with TBP and TFIIB are accessible to these general transcription factors. These regions may be

buried or otherwise inaccessible when C/EBPα binds to sites in the LSR and, alternatively, the region of C/EBPα that interacts with a co-regulator may be readily accessible only when C/EBPα occupies sites within the LSR. These data highlight a new level of complexity regarding the mechanism transcription factors use to diversify hormonal responses.

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